DIAGNOSTIC METHODS FOR EPIDEMIOLOGICAL STUDIES OF TROPICAL THEILERIOSIS (*THEILERIA ANNULATA* INFECTION OF CATTLE)

TÜLÍN ÍLHAN (KARAGENÇ)

DOCTOR OF PHILOSOPHY
UNIVERSITY OF EDINBURGH
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DECLARATION

I hereby declare that the work presented in this thesis is the product of my own efforts except where stated in the text or acknowledgements.

Tülin Ilhan (Karagenç)

DEDICATION

To my parents,

With love and thanks

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ABBREVIATIONS

APS	ammonium persulphate
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BoLA	bovine leukocyte antigen
bp	base pair
C++	high positive serum control
C+	low positive serum control
C-	negative serum control
Cc	conjugate control
cDNA	complementary deoxyribonucleic acid
CI	confidence intervals
CTVM	Centre for Tropical Veterinary Medicine
dATP	2'-deoxadenosine 5'triphosphate
dCTP	2'-deoxycytidine 5'triphosphate
dGTP	2'-deoxyguanosine 5'triphosphate
dTTP	2'-deoxythymidine 5'triphosphate
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immuno-sorbent assay
GST	glutathione S-transferase
HRPO	horse radish peroxidase
IFAT	immunofluorescent antibody test
Ig	immunoglobulin
IPTG	isopropythio-β-D galactosidase
kb	kilo base
kDa	kilo Dalton
LB	L- Broth medium
LCL	lower control limit
mAb	monoclonal antibody
NBT	nitroblue tetrazolium
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PBS/0.05T	phosphate buffered saline with 0.05% Tween-20
PCR	polymerase chain reaction
PCV	packed cell volume
Pfu	plaque forming unit
PMSF	phenylmethylsulfonyl fluoride
PP	percent positivity
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid

ssu rRNA	small subunit ribosomal RNA
SDS	sodium dodecyl sulphate
SDS- PAGE	SDS-polyacrylamide gel electrophoresis
SSC	sodium chloride and sodium citrate buffer
TBE	tris-HCl, boric acid and EDTA
TEMED	N,N,N',N', - tetramethylethylenediamine
TG-ROC	two graphs receiver operating characteristic
TMB	tetramethylbenzidine dihydrochloride
UCL	upper control limit
UV	ultra-violet illumination
WBC	white blood cells
X-gal	5-bromo-4 chloro 3 indolyl β-D galactosidase

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ABSTRACT

The aim of this study was to develop improved diagnostic techniques for tropical theileriosis caused by *T. annulata.* These were (i) stage specific indirect enzyme-linked immunosorbent assays (ELISA) to distinguish animals vaccinated with high passage cell lines from those naturally infected in the field and (ii) a highly sensitive and specific polymerase chain reaction (PCR) methodology to detect low level infections in carrier animals.

Material for this study was provided by experiments in which calves were infected in different ways to simulate the field situation. Three groups of animals were infected with different life cycle stages of *T. annulata:* sporozoites, a low passage macroschizont-infected cell line or a high passage (attenuated) cell line, normally used as a vaccine to control tropical theileriosis, and challenged with virulent heterologous sporozoites. The challenge indicated that different parasite stages and levels of attenuation used for primary infection stimulated different levels and duration of immunity. The immunity stimulated by sporozoites and the low passage cell line was solid throughout the study, in contrast to immunity induced by the high passage cell line which fell by seven months post-infection. The carrier status of these animals was determined by PCR using primers from small subunit ribosomal RNA (ssu rRNA) in comparison to that illustrated by microscopic detection of piroplasms and cell culture isolation of macroschizonts. The different patterns revealed that calves infected with sporozoites or the low passage cell line were persistent carriers, while those inoculated with the high passage cell line were intermittent or only transient carriers.

In order to obtain an antigen expressed by the macroschizont stage of the parasite to use in ELISA an attempt was made to isolate a *T. annulata* gene, which was a homologue of the QP rich protein of *T. parva,* from a *T. annulata* ZAP cDNA library. Three *T. annulata* genes, NC1, NC2 and NC10, were isolated from the cDNA library by screening with QP cDNA probe. Characterisation of these genes was carried out by Southern, northern, western blot and sequence analyses. An immunogenic recombinant protein, NC10-Ssp13, was obtained from the NC10 gene which was expressed by the

macroschizont stage of the parasite. Antisera against the NC10-Ssp13 antigen detected polypeptides both in macroschizonts and host nucleus.

Indirect ELISA using recombinant antigens, a merozoite rhoptry recombinant antigen (Tamr-1), NC10-Ssp13 and an antigen expressed by both the macroschizont and piroplasm stages of the parasite, Tash-2, were standardised following international guidelines on data expression and quality assurance. The results were compared with those obtained with indirect immunofluorescence antibody test (IFAT) using piroplasm antigen. The recombinant antigens, Tamr-1, NC10-Ssp13 and Tash-2, were screened against antisera produced by infection of cattle with different life cycle stages of *T. annulata* in order to examine whether the combination of the Tamr-1 and NC10-Ssp13 ELISAs or Tamr-1 and Tash-2 ELISAs would distinguish vaccinated animals from those that are naturally infected. The result showed that both Tamr-1 and Tash-2 antigens sensitively detected the antibodies in animals infected with both sporozoites and the low passage cell line. No antibody responses were detected against the Tamr-1 antigen, and were very low against the Tash-2 antigen in animals immunised with the high passage cell line. The antibody responses detected by the NC10-Ssp13 ELISA were lower than those obtained for both Tamr-1 and Tash-2 ELISAs.

The results of this study show that, ELISAs using the Tamr-1, NC10-Ssp13 or Tash-2 recombinant antigens did not reliably distinguish animals vaccinated with the high passage cell line from those that were naturally infected. Both Tamr-1 and Tash-2 ELISAs, however, could serve as powerful diagnostic tools in epidemiological studies of theileriosis, as ELISA systems permit processing of larger numbers of samples than IFAT and give objective results.

The nested PCR using primers derived from the gene encoding the 30 kDa major merozoite surface antigen (Tams-1) of *T. annulata* amplified parasite DNA in blood samples from animals exhibiting low piroplasm parasitaemia. The PCR distinguishes *T. annulata* from *T. buffeli* in cattle and *T. annulata* from *T. lestoquardi* and *B. equi* in vector ticks. Such sensitive and specific tests would complement the sero-epidemiological studies of theileriosis and help to target the vaccine produced.

CHAPTER ONE

INTRODUCTION

Tropical theileriosis is an important and frequently fatal disease of cattle caused by the protozoon parasite *T. annulata* (Dschunkowsky and Luhs, 1904). It is transmitted by ixodid ticks of the genus *Hyalomma* (Robinson, 1982). The disease occurs in the Mediterranean littoral, North Africa, the Middle East, the Indian subcontinent and Central Asia where an estimated 250 million cattle are at risk (Purnell, 1978). In particular tropical theileriosis threatens exotic *Bos taurus* breeds of European origin and their cross-bred progeny in which it may cause 40-60% mortality (Brown, 1990). When cattle survive infection recovery is extended and often incomplete resulting in loss of productivity and a carrier state (Uilenberg, 1981b). Therefore, bovine tropical theileriosis imposes serious constraints upon breed improvement programmes and livestock production.

The control of tropical theileriosis is based primarily on vector control, chemotherapy and, in many countries, vaccination using attenuated macroschizont infected cell lines. The control of disease in Turkey other countries from North Africa to China has been carried out by immunisation using an attenuated macroschizont infected cell culture vaccine (Onar, 1989). 150,000 doses of vaccine are produced each year at the State Veterinary Institute, Pendik, near Istanbul in Turkey. This vaccine has been used to protect particularly valuable, highly susceptible cattle in over 60 urban and peri-urban milk-producing areas in Turkey (Onar, 1989). Despite the vaccine programme and the more recent development of treatment with buparvaquone, theileriosis has become an increasing problem in recent years due to the increase in cross-breeding and the demand for exotic cattle to improve both dairy and beef production. Consequently the annual production of vaccine is no longer sufficient to fulfill the requirement of Turkey. Not only is larger scale production needed, on a sustainable, cost recoverable basis, but the vaccine needs to be shown to be effective and to be targeted where it is most needed.

There are a number of factors that must be determined in the design of a successful vaccination programme: (i) the proportion of hosts that must be

vaccinated, (ii) the age at which hosts should be first vaccinated, (iii) at what interval hosts should be re-vaccinated (Woolhouse *et al.*, 1997). All these require comprehensive epidemiological knowledge of the disease that can only be obtained using diagnostic tools that sensitively and specifically detect the presence of parasite or antibodies which recognise parasite antigens.

In order to assess the impact of a *T. annulata* control programme, it is necessary to assess whether an animal has been vaccinated effectively i.e. the vaccine 'take', whether it has been challenged and whether it is immune to infection by *T. annulata.* One way of measuring the effectiveness of attenuated cell line vaccines is by monitoring the antibody response to the parasite as a consequence of vaccination. To date the most common method used to monitor the response to theilerial vaccines is the indirect immunofluorescent antibody test (IFAT) using both macroschizont and piroplasm antigens (Pipano and Cahana, 1969; Anon, 1997). However, IFAT fails to distinguish vaccinated/unchallenged animals from those naturally infected because of the presence of common antigens in all stages of the parasite (Knight *et al.*, 1998).

The attenuated cell line vaccine used in Turkey has been reported not to produce piroplasms in immunised cattle (Ozkoc and Pipano, 1981). Therefore, using stage specific antigens might allow vaccinated animals to be distinguished from those that are naturally infected. During natural infection with sporozoites, the host immune system will be exposed to sporozoites, macroschizonts, merozoites and piroplasms. In contrast, animals immunised with an attenuated high passage cell line vaccine, which does not produce piroplasms, will be exposed only to the macroschizont stage of the parasite. Thus, a macroschizont specific antigen could be used to assess the vaccination status of cattle. Whereas a sporozoite or merozoite /piroplasm stage specific antigen could be used to detect challenge or natural infection in the field. These antigens would be of great value in investigating the true prevalence of infection where the vaccine has already been used and in assessing vaccination programmes.

In recent years, stage specific antigens of the different life cycle stage of *T. annulata* have been expressed as recombinant proteins. These include the sporozoite surface antigen, SPAG-1 (Hall *et al.*, 1992; Williamson *et al.*, 1994), the merozoite

rhoptry antigen, Tamr-1 (Shiels *et al.*, 1994), and the 30 kDa major merozoite and piroplasm surface antigen, Tams1 (Shiels *et al.*, 1995). Preliminary studies demonstrated that antibodies against the Tamr-1 antigen were detected in animals infected with sporozoites or with non-attenuated macroschizont infected cells, whereas the antibody titres of animals immunised with high passage cell lines remained close to the pre-immunisation levels (Ilhan, 1995). On commencement of this study there were no recombinant antigens available which were expressed by the macroschizont stage of *T. annulata.* Such an antigen was needed for the development of a diagnostic test aimed at the measurement of efficacy of vaccination. Using such antigens in an enzyme-linked immunosorbent assay (ELISA) systems would hopefully provide a tool to distinguish vaccinated from naturally infected animals. Additionally, an antibody ELISA has several advantages over IFAT including the ability to test large numbers of samples rapidly and economically, thus making it relevant to the conduct of large scale sero-epidemiological surveys.

Another important aspect of *T. annulata* infection is that a persistent piroplasm parasitaemia occurs in the host following recovery from natural infection (Sergent *et al.*, 1945; Neitz, 1957). Vector ticks, which have fed on carrier cattle and become infected, have the potential to transmit the parasite to a susceptible host. Therefore, the carrier state is of great importance in the maintenance of the life cycle of the parasite by alternate tick/cattle passage. The carrier state has, in the past, been determined by microscopic examination of Romanowsky stained blood smears for the presence of piroplasms. Nevertheless, piroplasm parasitaemia is often too low to be detected in carriers and can be misdiagnosed where the distribution of other *Theileria* species is sympatric with *T. annulata* (Norval *et al.*, 1992; Uilenberg, 1981b). This results in underestimation or overestimation of the true prevalence of the carrier state in a population. With advances in molecular biology, the detection of very low number of parasites in the host and vector has become possible. One of the most frequently used methods is the polymerase chain reaction (PCR) using parasite specific oligonucleotide sequences. Recent studies showed that PCR using primers from the small subunit ribosomal RNA (ssu rRNA) gene (Allsopp *et al.*, 1993; de Kok *et al.*, 1993; Ilhan, 1995) or the gene encoding a 30 kDa merozoite surface protein (Tams1) (Shiels *et al.*, 1995; d'Oliveira *et al.*, 1995; 1997b) detected

T. annulata sensitively and specifically in both the vertebrate host and tick vectors. An effective PCR would permit simple, practical studies of the carrier state of animals in the field. Such studies are needed for an accurate assessment of theileriosis in risk areas, and as a prerequisite for effective targeting of vaccines for the control of tropical theileriosis.

The primary objectives of this study were to develop improved diagnostic techniques. These were: i) stage specific indirect ELISAs to distinguish animals vaccinated with high passage cell lines from those naturally infected in the field and ii) a highly sensitive and specific PCR methodology to detect low level infections in carrier animals and in vector ticks. The work was carried out in the following stages:

1) Design controlled animal experiment that simulates the field situation in order: a) to produce materials for development of stage specific ELISAs, b) to evaluate, within the time available, duration of immunity, c) to examine the relationship between the carrier state of cattle and immunity to challenge.

2) Clone a macroschizont gene of *T. annulata* and express it as a recombinant protein for use in an ELISA.

3) Determine the immunogenicity of the Tamr-1 and macroschizont specific antigens, and establish the diagnostic sensitivity and specificity of the Tamr-1 and macroschizont ELISAs for detection of antibodies against *T. annulata.*

4) Assess the Tamr-1 and macroschizont ELISAs in the context of their ability to distinguish vaccinated animals from those naturally infected and thus determine their value as diagnostic tools for epidemiological studies.

5) Develop a highly sensitive and specific PCR methodology to detect low level infections in carrier animals.

CHAPTER TWO

LITERATURE REVIEW

The aim of this study was to develop improved diagnostic techniques for tropical theileriosis. Thus, this review concentrates on the parasite, the disease it causes, immune responses to disease, control measures and diagnostic techniques that have been used. In addition, since this study is focused on the control programme carried out using attenuated cell line vaccine in Turkey, epidemiology of tropical theileriosis and studies on disease in Turkey are briefly described.

2.1. IDENTIFICATION AND CLASSIFICATION OF *THEILERIA ANNULATA*

Dschunkowsky and Luhs (1904) encountered a fatal disease of cattle in Transcaucasia, in the former Imperial Russia, which they named "Tropical Piroplasmosis". Cattle infected with this disease had oval or round erythrocytic parasite forms similar but not identical to the rod shaped parasites observed by Koch (1898) in East Coast fever. This parasite was then named *Piroplasma annulata* (Dschunkowsky and Luhs, 1904). The presence of the schizont stage in the life-cycle of *P. annulata* contributed to its removal from the genus *Piroplasma* and placement in the genus *Theileria* (Bettencourt *et al.*, 1907). In the following decades diseases associated with *T. annulata*-like parasites were reported. Lack of knowledge and defined reference stocks often resulted in their misdiagnosis as *T. parva.* In addition many "new" *Theileria* species were reported such as *T. dispar, T. turkestanica* and *T. sergenti* which were frequently confused with *T. annulata*. In his review, Neitz (1957) stated that *T. annulata* and these species were synonyms. The former two species were almost certainly *T. annulata*, but controversy still encompasses the identity of *T. sergenti.*

The taxonomy of the *Theileria* genus at species level has been controversial and is still not satisfactorily resolved. The classification suggested by Levine (1988) is as follows:

Subkingdom	Protozoa	
	Phylum	Apicomplexa
	Class	Sporozoea
	Order	Piroplasmorida
	Family	Theileriidae
	Genus	*Theileria*
	Species	*Theileria annulata* (Dschunkowsky and Luhs, 1904).
		T. parva (Theiler, 1904) and others

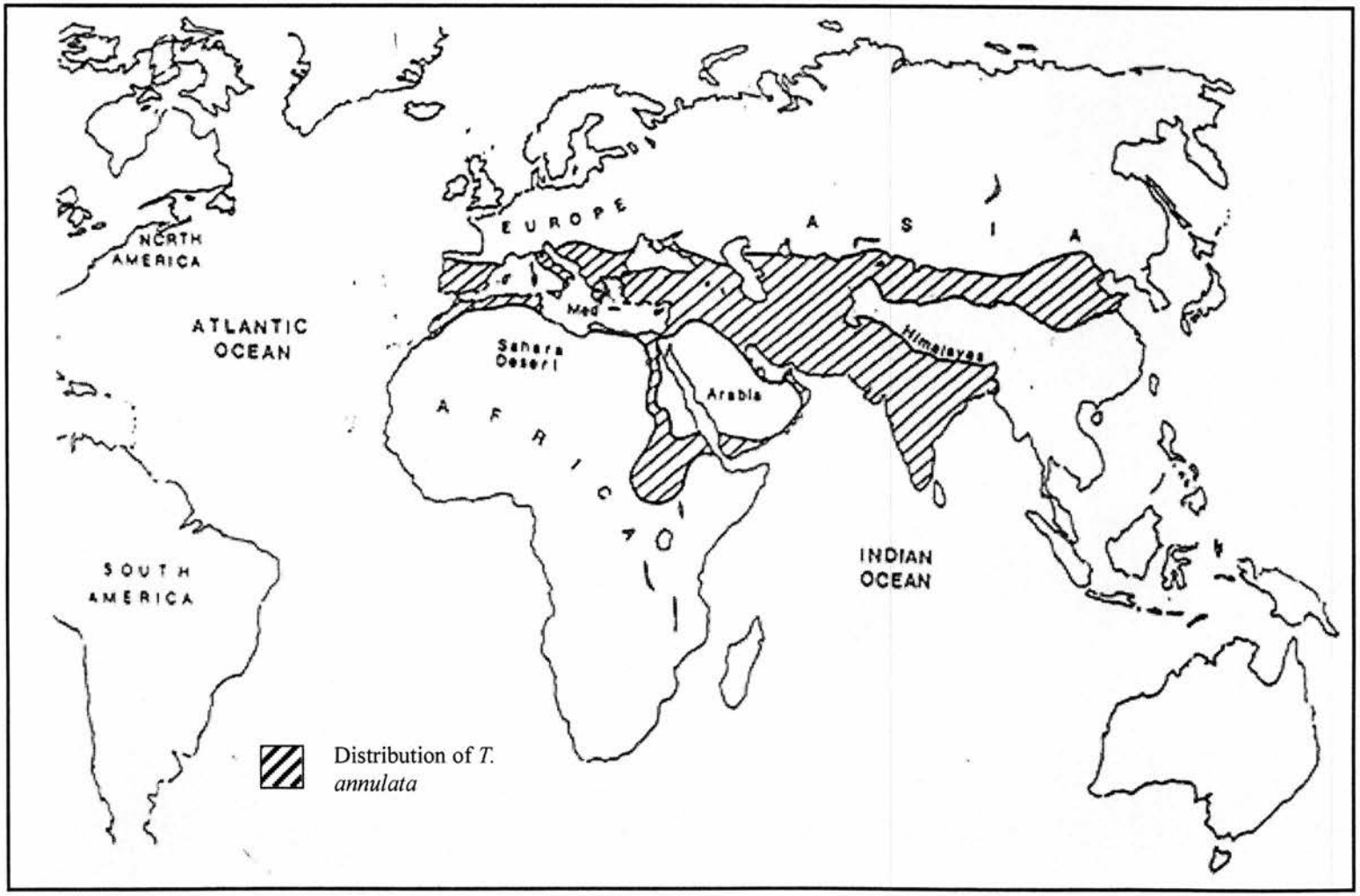
2.2. DISTRIBUTION OF TROPICAL THEILERIOSIS

T. annulata, associated with ixodid ticks of the genus *Hyalomma* is widespread in cattle from Southern Europe, North Africa extending into Northern Sudan, and the Near and Middle East, including India, Central Asia (Purnell, 1978) and Northern China (Lu and Yin, 1994). Dolan (1989) suggested that where the distribution has been described as extending eastward from India to the Pacific coast including Southern China and Far Eastern countries south of China into Malaysia, the disease probably incorporates the distribution of *T. sergenti/T. orientalis*. Figure 2.1 shows the probable distribution of tropical theileriosis.

The distribution and seasonal occurrence of *T. annulata* is restricted by the geographical location and biology of its transmission vector, ixodid ticks of the genus *Hyalomma* (reviewed by Robinson, 1982). In most endemic areas the majority of cases occur between June and September (Sergent *et al.*, 1945; Pipano, 1976) although sporadic cases may be found throughout the year (Flach and Ouhelli, 1992).

Susceptible domestic cattle (*Bos taurus* and *Bos indicus)* in areas where the tick vector occurs are, in principle, at risk of contracting tropical theilerosis. It is thought that Asian buffaloes (*Bubalus bubalis*) may be the original hosts and cattle secondary hosts (Uilenberg, 1981b). The disease is mild in buffalo, but highly pathogenic to cattle, especially non-indigenous breeds. Steuber *et al.* (1986) examined the host range of *T. annulata in vitro* and demonstrated that the sporozoites neither entered nor attached to peripheral blood mononuclear cells (PBM) from horse, rabbit, mouse, hamster or man. Recently, it was shown that *T. annulata*

Figure 2.1. The geographical distribution of *T. annulata* (E. Kirvar).


NORTH AMERICA
EUROPE
A S I A
ATLANTIC OCEAN
Med
Sahara Desert
Arabia
Himalayas
A F R I C A
SOUTH AMERICA
INDIAN OCEAN
Distribution of *T. annulata*

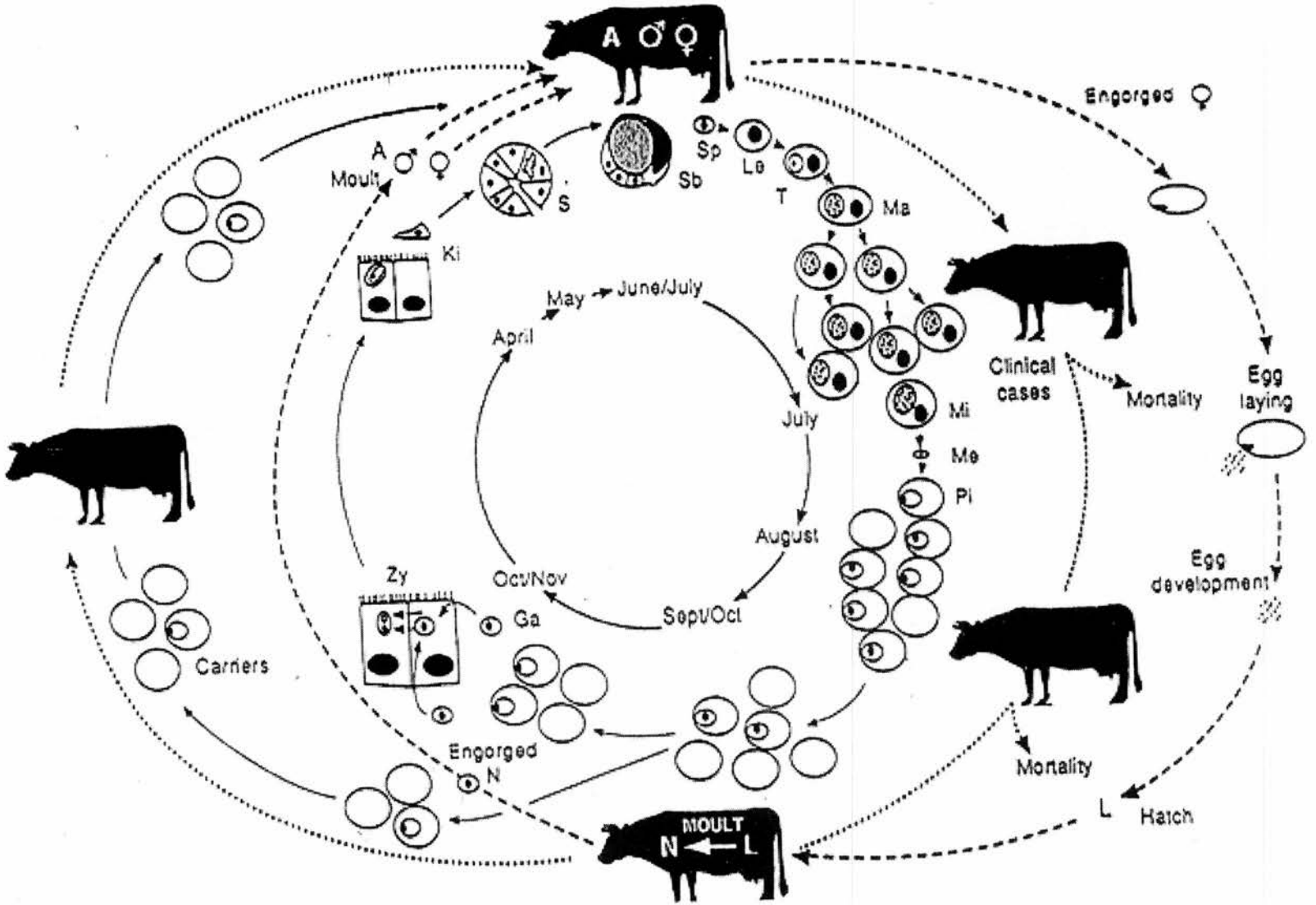
sporozoites infects PBM cells of goats and sheep both *in vivo* and *in vitro* (Brown *et al.*, 1998a; Leemans *et al.*, 1998). In goat and sheep *T. annulata* sporozoites produce macroschizonts *in vivo*, but do not produce piroplasms (Leemans *et al.*, 1998). These studies confirmed that the host range of *T. annulata* is limited to the bovid family.

T. annulata is maintained in nature by a cattle-tick-cattle cycle. *T. annulata* may complete its life-cycle in ticks on pasture or in barns. Engorged nymphs of *H. detritum*, one of the main vectors of *T. annulata*, hide in the cracks and crevices of clay or barn walls and, after moulting, adults infect cattle introduced into these barns (Pipano, 1994). The hypothetical synchrony between parasite, two-host *H. detritum* tick and bovine host life-cycle in North Africa, for example, under seasonal conditions is illustrated graphically in Figure 2.2 (Flach and Ouhelli, 1992). It appears that the *T. annulata* cycle proceeds exclusively within cattle during the summer but divides into two pathways in the autumn so that the parasite over-winters either as piroplasms and possibly schizonts in carrier cattle, or as zygotes in engorged nymphal ticks. This follows sexual replication in the gut of the engorged nymph (Flach and Ouhelli, 1992). Subsequently, there is further development to the kinete and then sporogony occurs with nymphs moult to adults. Attachment and feeding of the adult in the spring and early summer ensures further transmission of the parasite to the bovine host (Flach and Ouhelli, 1992).

2.3. THE VECTORS OF *T. ANNULATA* AND ITS TRANSMISSION

Sergent *et al.* (1928) demonstrated for the first time that *T. annulata* in Algeria was transmitted by an ixodid tick, *Hyalomma mauretanicum* (*H. detritum,* Schulze, 1919). The development stages undergone by the parasite within the tick vector was shown by the same authors (Sergent *et al.*, 1936). Several later studies have shown that *T. annulata* is naturally or experimentally transmitted by 15 species of the genus *Hyalomma* (Robinson, 1982). The major vectors appear to be the three host tick *H. anatolicum anatolicum* and the two host tick *H. detritum* (Uilenberg, 1981a). The former is widely distributed in Northern Africa, the Near East, Asia Minor and the Southern ex-Soviet Union States to the Indian Subcontinent whereas *H. detritum* is found in warmer regions from Manchuria through China and India to north-west Africa (Norval *et al.*, 1992).

Figure 2.2. Hypothetical synchronised life cycles of *T. annulata* and *H. detritum* and their interaction with cattle (Flach and Ouhelli, 1992). Thin solid line: *T. annulata;* thick broken line *H. detritum,* thin broken line cattle.
Abbreviation for *T. annulata*: Le: leukocyte, T: trophozoite, Ma: macroschizont, Mi: microschizont, Mc: Mcrozoite, Pi: piroplasm, Ga: gamate, Zy: zygote, Ki: Kinete, S: salivary gland, Sb: sporoblast, Sp:sporozoites.
Abbreviation for tick: L: larvae, N: nymph, A: adult.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June/July
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Clinical cases
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The parasite is transmitted transtadially by these vector ticks. In the two host tick, in which larvae and nymphs feed on the same animal, only the adult can be infective whereas in the three-host species, nymphal or adult stages may be infective (Uilenberg, 1981a). Tick species whose immature form normally feed on non-susceptible hosts such as small mammals and birds do not transmit the disease. For example *H. excavatum* transmits *T. annulata* experimentally, but in many areas its immature forms normally feed on rodents and therefore the adults are unable to transmit the disease (Barnett, 1977).

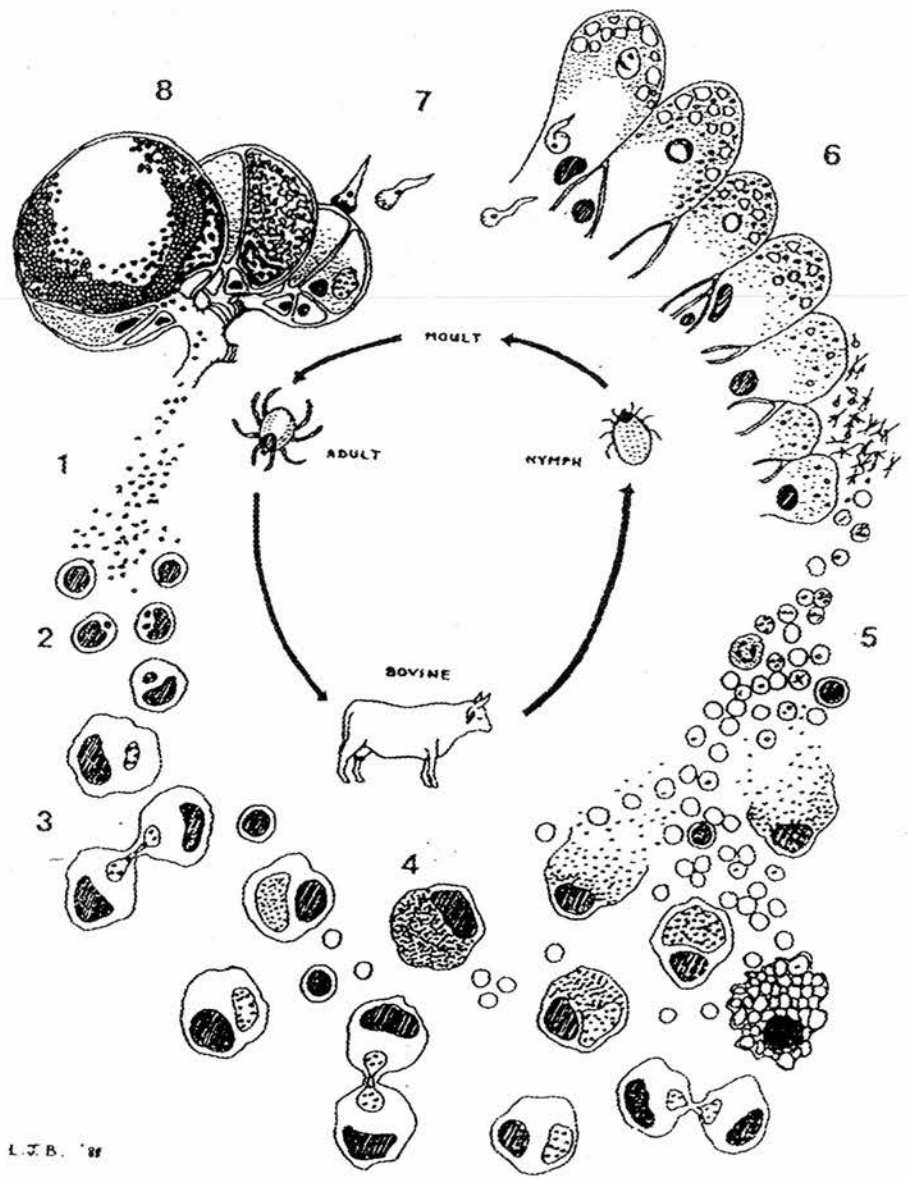
2.4. THE LIFE-CYCLE OF *THEILERIA ANNULATA*

The basic features of the life cycle of the parasite in the bovine and tick hosts are illustrated in Figure 2.3. The life cycle of the parasite can be divided into three main stages; schizogony in the bovine host, gametogony and sporogony in the tick host.

The life cycle of *T. annulata* starts when an infected tick takes a blood meal, resulting in the introduction of sporozoites into the host blood stream. Recently, the biology of *Theileria* sporozoite invasion of bovine cells was reviewed by Shaw (1997). The sporozoites rapidly enter mononuclear cells (Fawcett *et al.*, 1982; Jura *et al.*, 1983b). *T. annulata* sporozoites preferentially target cells displaying major histocompatibility complex (MHC) class II molecules: the macrophage/monocyte lineage and at a much lower efficiency, B cells (Glass *et al.*, 1989; Spooner *et al.*, 1989; Campbell *et al.*, 1994b). Sporozoites gain entry into lymphocytes within 5-60 minutes of inoculation by receptor mediated endocytosis, and as many as 15 sporozoites can be internalised within an individual cell. Sporozoite entry occurs through a defined series of separate but contiguous events, namely initial binding, zippering and internalization, and the escape of the parasite into the host cell cytoplasm (Fawcett *et al.*, 1982; Jura *et al.*, 1983a; Jura *et al.*, 1983b; Shaw *et al.*, 1991).

The sporozoite is nonmotile and initial binding to the lymphocyte occurs through a passive process. However the subsequent entry into the cell is active (Shaw *et al.*, 1991). While sporozoite binding is a temperature independent and can occur at 0-2°C, all subsequent steps in invasion the process are temperature dependent (Jura,

Figure 2.3. Life cycle of *Theileria annulata.* 1.sporozoites entering bovine host tick saliva, 2. trophozoites in mononuclear cells, 3. macroschizonts in transformed mononuclear cells, 3. merozoites in transformed mononuclear cells, 4. piroplasms in erythrocytes, 6. gamogony cycle in tick gut cells, 7. kinetes in haemolymph, 8. sporogony cycle in tick salivary gland. (Figure prepared by Leslie Bell-Sakyi)


8
7
6
MOULT
ADULT
NYMPH
1
2
5
BOVINE
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L.J.B. '88

1984; Shaw *et al.*, 1991). Entry is accomplished by a progressive circumferential zippering process characterised by the close apposition of the parasite and host cell membrane. Subsequent to internalisation the parasite escapes rapidly from the enclosing host cell membrane to lie free in the cytoplasm (Shaw, 1997). Escape into the host cytoplasm occurs concomitantly with the discharge of the rhoptries and microspheres (Fawcett *et al.*, 1982; Shaw *et al.*, 1991). A thick layer of fuzzy material then appears on the surface of the parasite to which the host cell microtubule arrays rapidly become associated (Fawcett *et al.*, 1982; Fawcett *et al.*, 1984; Shaw *et al.*, 1991).

Following entry, the sporozoite develops into a uninucleate trophozoite stage (Jura *et al.*, 1983a). The trophozoite undergoes a series of nuclear divisions and forms a syncytial macroschizont with between 10-20 nuclei. The macroschizont lies free within the host cell cytoplasm and is surrounded by a plasma membrane that has no obvious outer surface coat (Shaw and Tilney, 1992). The nuclei are scattered randomly throughout the cytoplasm and surrounded by a typical nuclear envelope with nuclear pores. A unique event occurs following development of macroschizonts as *Theileria*-infected cells become immortalised and the infected cells can be propagated indefinitely *in vitro* without requirement for exogenous growth factor (Brown, 1987). The host cell and parasite divides synchronously (Hulliger *et al.*, 1964). The transformed host cells divide at regular intervals and at a rapid rate *in vitro*, which often gives rise to a 10-fold increase in infected cells every three days or less (Irvin *et al.*, 1982). During host cell division, the schizonts also divide and distribute to each daughter cells by a process which involves their attachment to the spindle apparatus (Hulliger *et al.*, 1964; Stagg *et al.*, 1980; Mehlhorn and Schein, 1984; Carrington *et al.*, 1995). Infected cells can be detected in the lymph node draining the site of the tick bite from about 5 days after infection.

During the course of infection, a proportion of the schizonts undergo merogony to produce large numbers of merozoites (Shaw and Tilney, 1992). Merogony is characterised by a series of structural and organisational changes. These changes include development of smooth and rough endoplasmic reticulum, and the formation of an external coat on the outer surface of the schizont plasma membrane, which is also found on the surface of the mature merozoites. Nuclei migrate to the

periphery of the schizont and uninucleate merozoites start to form. Rhoptries appear in the schizont cytoplasm in small clusters associated with the nuclear spindle pore bodies and fibrous structures beneath the plasma membrane. Mitochondria become closely associated with the outer membrane of the nuclear envelope. Merozoites bud from the syncytial schizont in a synchronous process. At the end of merogony the host cell contains large numbers of mature merozoites that are liberated by the breakdown of the host cell plasma membrane (Shaw and Tilney, 1992).

The merozoites become free and penetrate the erythrocytes to form piroplasms. Merozoites can be detected 8-10 days after infection with *T. annulata* and 13 days with *T. parva.* In extreme cases up to 90% of erythrocytes may become infected with *T. annulata* (Mehlhorn and Schein, 1984). Merozoite entry into bovine erythrocytes proceeds by a process morphologically similar to that described for sporozoite entry into mononuclear cells (Shaw and Tilney, 1992). Following entry, the parasite escapes from the surrounding host membrane into the host cytoplasm and discharges the rhoptries and/or microspheres. The piroplasm lies free in the cytoplasm of the erythrocyte but, unlike the macroschizont, host cell tubules do not associate with the surface of the merozoite. Conrad *et al.* (1985; 1986) and Fawcett *et al.* (1987) suggested that division of merozoite and piroplasm was by schizogony of the spherical forms of piroplasm to produce maltese-cross forms similar to the small *Babesia spp* (previously *Nuttalia)* such as *B. microti* and *B. equi* (*Theileria equi*) (Schein *et al.*, 1981; Moltman *et al.*, 1983). The merozoites released from the erythrocytes are identical to those released from schizont infected lymphocytes, which has led to the suggestion that these forms could be responsible for the re-invasion of erythrocytes (Conrad *et al.*, 1985). When a tick feeds on an infected host, infected erythrocytes are taken up and transmission of parasite between host and vector occurs.

Infected erythrocytes ingested by the tick are lysed within the gut releasing the piroplasms of which a variable proportion undergo development into sexual stages (Norval *et al.*, 1992). Spherical gamonts from the infected blood differentiate into ray bodies. These bodies were first seen by Koch (1906) and rediscovered by Schein (1975). Ray bodies develop into multinucleated microgamonts and then uninucleated microgametes. These structures have up to four flagella-like projections

and a slender posterior pole. Other gamonts transform directly into macrogamonts (Gauer *et al.*, 1995). The haploid microgametes fuse with haploid macrogametes to form diploid zygotes (Gauer *et al.*, 1995) which enter tick gut epithelial cells. This stage and the succeeding kinete form are thought to be the only diploid forms during the parasite life cycle. Sexual reproduction within the tick was shown by Morzaria *et al.* (1992) who illustrated that recombinant parasites of mixed parental genotypes can be obtained from ticks fed on animals infected with two distinct parental stocks of *T. parva.* Following zygote formation, there are differences in the further development of *T. parva and T. annulata* life cycle (Gauer *et al.*, 1995). The zygotes of *T. parva* and *T. annulata* replicate and become tetraploid. The zygotes of *T. parva* then undergo a two-step meiosis in the tick gut epithelium of their vector tick. Following meiosis *T. parva* zygotes are reduced to a haploid set and kinetes developing from this haploid zygotes initially show haploid DNA contents before they increase their DNA to a polyploid level. Whereas *T. annulata* zygotes seems to be increase to polyploid level during the process of differentiation into kinetes (Gauer *et al.*, 1995). The zygotes transform into club-shaped motile kinetes that penetrate the gut epithelial cells of the tick and migrate to the salivary glands via haemolymph (Mehlhorn and Schein, 1984). The formation of infectious sporozoites in the acinar cells of the tick salivary gland are similar for *T. annulata* and *T. parva.* The single kinete transforms into a sporoblast and undergoes multiple division. At this stage maturation is delayed until sporogony is stimulated by tick feeding or high temperature incubation (Samish, 1977). The parasitised type II and type III salivary gland acini (Schein and Friedhoff, 1978) undergo marked enlargement due to the parasite forming a multinucleate syncytium occupying most of the cell cytoplasm (Fawcett *et al.*, 1985). During tick feeding there is a rapid development of the syncytium which gives rise to thousands of uninucleate sporozoites by cytoplasmic fission (Mehlhorn and Schein, 1984). It is at this stage that the resulting sporozoites are haploid (Gauer *et al.*, 1995). The formation of uninuclear sporozoites occurs around day 3 of feeding or day 4 of heat stimulation (Reid and Bell, 1984).

2.5. THE ECONOMIC IMPORTANCE OF TROPICAL THEILERIOSIS

It has been suggested that *T. annulata* is probably the most important of all the tick borne disease agents of domesticated livestock (Purnell, 1978). The disease is particularly important in breed improvement programmes (Rak, 1978) and the development of intensive dairy and beef units in tropical and subtropical areas infested with parasite (Robinson, 1982). It is estimated that around 250 million cattle are at risk of the disease in endemic regions. The disease is highly pathogenic to susceptible, exotic or improved cattle, causing 40-60% mortality (Brown, 1990).

Most indigenous cattle possess some natural resistance to theileriosis, due to long associations between these cattle and the parasite, which helps to produce a state of endemic stability (Anon, 1991). The devastation of the disease is seen dramatically when exotic or 'improved' crossbred cattle are introduced to tick-infected areas. Animals exposed to the parasite either die or become immune and their immunity is maintained through their constant rechallenges. Furthermore, cattle recovering from infection suffer weight loss, fertility problems and diminished milk yields. The economic losses sustained include not only the price of expensive imported breeding animals but also the potential contribution of those animals to the genetic improvement of a national herd, with consequent loss in potential production (Anon, 1991).

Flach (1991) carried out a study in Morocco to estimate economic losses due to theileriosis including mortality, cost of treatment, reduced meat and milk production. This figure was £3,500 per 100 cattle each year on farms in endemic areas. The losses from *T. annulata* infection is estimated at US$ 800 million in India which compromises direct loses from death of animals and loss of productivity, and indirect losses due to the costs of control measures (Griffiths and McCosker, 1990; Brown, 1997a)

2.6. CLINICAL SIGNS AND PATHOGENESIS

The severity of the disease in cattle infected with *T. annulata* is dependent upon the susceptibility of an animal (Rafyi *et al.*, 1965), the virulence of the parasite strain (Sergent *et al.*, 1945; Pipano, 1989b) and the number of sporozoites inoculated (Preston *et al.*, 1992). The disease caused by *T. annulata* may be classified according

to its symptoms into mild, peracute, acute, subacute and chronic forms (Sergent *et al.*, 1945; Neitz, 1957).

In *T. annulata* infection, both the intracellular schizont stage and the intraerythrocytic piroplasm contribute to the pathogenesis (Irvin and Morrison, 1987). The incubation period (time between tick attachment and onset of fever) of disease is, on average, two weeks, with extremes of eight and thirty days (Sergent *et al.*, 1945; Neitz, 1957). One or two days before the febrile response, there is enlargement of the local lymph node draining site where infected ticks have been feeding, and biopsy smears of this node show hypertrophied lymphoid and monocytic cells and the presence of schizonts. The most common clinical symptoms of theileriosis are fever, inappetence, cessation of rumination, drooling from mouth, serous nasal discharge, swelling of the eyelids, lachrymation, accelerated pulse, drop in milk production and sometimes nervous symptoms (Khanna *et al.*, 1980). At the beginning of the pyrexial period the faeces are firm, but later there is diarrhoea often containing blood and mucous cast (Barnett, 1977). The animal becomes markedly emaciated.

The enlargement of the local drainage lymph node precedes the appearance of clinical signs of disease. Parasitised cells are detected in the regional lymph node from day 5 after inoculation of sporozoites in association with an increase in cell output (Irvin and Morrison, 1987). After the first appearance of macroschizonts on day 5 piroplasms can be detected in blood smears from day 9 after infection (C.G.D. Brown pers. comm). During the disease there is development of severe leucopaenia due to a decline in the number of circulating lymphocytes and neutrophils (Prasad, 1946; Laiblin, 1978; Preston *et al.*, 1992) and severe anaemia with a reduction of erythrocyte numbers occurring during parasitaemia crises. Anaemia develops with bilirubinaemia (Neitz, 1957). Anaemia becomes severe and animals develop pronounced dyspnoea. It has been demonstrated that up to 90% of erythrocytes can be infected by the parasites, and although direct lysis of infected cells by the parasite adds to the anaemia, it is believed that the removal of infected erythrocytes by the spleen and liver is the direct cause (Hooshmand-Rad, 1976; Barnett, 1977; Uilenberg, 1981a). Autoimmune mechanisms have been suggested as playing a role in pathogenesis of the anaemia in animals infected with *T. annulata* (Hooshmand-

Rad, 1976). The mortality rate in susceptible adult cattle is related to the virulence of the parasite stock and has been reported as between 40-60%. Death usually occurs between one and two weeks after the onset of clinical signs (Uilenberg, 1981a). Animals that recover from the infection become long-term carriers of the parasite (Sergent *et al.*, 1945).

The pathological lesions are mainly associated with lymphopoeitic and vascular systems (Sergent *et al.*, 1945; Neitz, 1957; Pipano, 1994). The severity of lesions is primarily dependent on the course of disease. The main macroscopic lesions are petechiae on subcutaneous tissues and serosa, hyperplasia of lymph nodes both internal and superficial, splenomegaly, yellowish enlarged liver with distended bladder, epi- and endocardial petechiae and ecchymoses, grey-white foci on the cortex of the kidney. The abomasum usually shows characteristic ulcers consisting of a central necrotic area surrounded by a haemorrhagic zone. Similar ulcers may be observed along the entire length of the small and large intestine. The lungs are frequently oedematous.

The schizont-infected cells rapidly disseminate from the lymph node draining the site of inoculation to distant lymph nodes (e.g., precrural, mesenteric and mediastinal) and to spleen and thymus through lymphoid tissues. The parasitized cells also spread into non-lymphoid organs, i.e. liver, kidney, lung, abomasum, adrenal glands and pituitary glands by day 7, in the brain by day 12 and in the heart by day 14 after an acute infection with *T. annulata* (Forsyth *et al.*, 1999). Metastasis of infected cells may be due to their expression of matrix metalloproteinases (MMPs) (Baylis *et al.*, 1992; Somerville *et al.*, 1998a) and of adhesison molecules CD2, CD11b, very late antigen-4 (VLA-4) and CD9 (Forsyth *et al.*, 1999).

2.7. IMMUNITY TO TROPICAL THEILERIOSIS

Animals which have recovered from a primary infection with *T. annulata* or which have been immunised by infection either with macroschizont-infected cell line or sporozoites (with or without subsequent treatment) are solidly immune to homologous challenge and frequently show good immunity to heterologous challenge (Barnett, 1963; Irvin and Morrison, 1987; Hall, 1988). Protective immunity to infection appears to be dependent upon a complex of immune responses

to *Theileria*. Following each life cycle differentiation event within an infected animal, the bovine immune system is exposed to different antigenic determinants. As a result both humoral and cell mediated immune systems are generated during the *T. annulata* infection (Tait and Hall, 1990; Boulter and Hall, 1999). Studies of the immune mechanisms in *T. annulata* infection have recently been reviewed by Preston *et al.* (1999), Boulter and Hall (1999) and Campbell and Spooner (1999).

2.7.1. Humoral Immune Response to *T. annulata* infection

Following recovery, animals exhibit antibodies that react with each of the vertebrate stages of the parasite: sporozoites, macroschizonts, merozoite and piroplasms. Two lines of evidence from experiments, however, suggest that antibodies against the macroschizont and piroplasm stages of *T. parva* do not play a role in protective immune response. First, animals immunised with extracted schizont or piroplasm antigens had developed similar antibody responses to those observed in animals immunised by sporozoite infection (Wagner *et al.*, 1974). These animals were fully susceptible to challenge. Second, antibodies from immune animals do not react with the surface of either macroschizont infected cells (Shiels *et al.*, 1989) or the piroplasm infected erythrocytes (Ahmed *et al.*, 1988).

However, humoral responses are effective against extracellular stages of the parasite life-cycle, the sporozoite and merozoite. Gray and Brown (1981) demonstrated that sera taken from calves after multiple infections with *T. annulata* sporozoites inhibited entry of sporozoites into naive PBM *in vitro*. In the same study it was also demonstrated that *T. annulata* immune serum raised against one stock of *T. annulata* was able to neutralise the infectivity *in vitro* of heterologous sporozoite stock (Gray and Brown, 1981). Further research showed that serum from calves exposed repeatedly to sporozoite challenge also delayed the development of trophozoites to macroschizonts *in vitro* (Preston and Brown, 1985). The blocking effect of sporozoite invasion of host cells is only detectable after repeated sporozoite challenge (Preston and Brown, 1985; Williamson *et al.*, 1989). Since the sporozoites invade the cells in a very short time (Brown *et al.*, 1978b; Jura *et al.*, 1983a), it was inferred that high levels of circulating antibody will be needed to provide protection. In the field this would require by repeated challenges by infected ticks.

The observation that serum from hyperimmunised cattle neutralised the infectivity of sporozoites *in vitro* prompted studies to determine the nature of the target antigens. A series of monoclonal antibodies (mAbs) was raised against purified sporozoites. Some of these mAbs showed effective inhibition of sporozoite infectivity *in vitro* in both *T. annulata* (Williamson *et al.*, 1989) and *T. parva* (Dobbelaere *et al.*, 1984; Musoke *et al.*, 1984). One of the mAbs, 1A7, against *T. annulata* sporozoites recognised a series of polypeptides in sporozoite extracts of *T. annulata* which are thought to be the result of proteolytic processing of a single gene product (Williamson, 1988; Williamson *et al.*, 1989). Immunoelectron microscopical studies indicated that the antigen (SPAG-1), recognised by mAb 1A7, is located on the surface of the sporozoite (Knight *et al.*, 1996). Studies were carried out to characterise and assess the immunological significance of SPAG-1 of *T. annulata*, (Knight, 1993; Knight *et al.*, 1996; 1998; Boulter *et al.*, 1994; 1995). The gene encoding SPAG-1 was found to contain regions with a high degree of homology to a bovine elastin repeat sequence (Hall *et al.*, 1992). The presence of these repeats led to the suggestion that sporozoites may be using mimicry to bind to the bovine elastin receptors as means of invading cells and host recognition. However, this region was demonstrated to be polymorphic (Katzer *et al.*, 1994) and sporozoites were found to invade cells lacking the elastin receptor (Campbell *et al.*, 1994b). It has been shown that there are several immunodominant sites on the SPAG-1 molecule (Knight *et al.*, 1996), and there are neutralising B-cell epitopes which lie towards the C-terminus defined by immune calf serum (Boulter *et al.*, 1994). This molecule is a candidate for inclusion in a sub-unit vaccine and partial protection has been achieved in calves immunised with recombinant SPAG-1 (Boulter *et al.*, 1995; 1998; 1999).

Two different antigens, SPM1 and SPM2, both recognised by sporozoite-neutralising mAb 4B11, have been characterised (Knight, 1993; Knight *et al.*, 1998). Unlike SPAG-1, these antigens are also expressed by the schizont and piroplasm stages. However, their protective significance is not known.

Monoclonal antibodies neutralising sporozoite infectivity have also been identified for *T. parva* (Dobbelaere *et al.*, 1984; Musoke *et al.*, 1984). These mAbs recognise a 67 kDa protein expressed on the surface of the sporozoites (Iams *et al.*, 1990a; Nene *et al.*, 1992). Immunisation trials using recombinant p67 resulted in

complete or partial protection in a proportion of animals (Musoke *et al.*, 1992; Musoke *et al.*, 1993). The other fully characterised antigen from the sporozoite stage of *T. parva is* a 104 kDa microneme-rhoptry protein (Iams *et al.*, 1990b). However, antibodies to this molecule do not neutralise sporozoite invasion *in vitro* and vaccination with this antigen does not produce any protection (Morzaria cited in Boulter and Hall, 1999).

Limited studies have been conducted on the immune response against the merozoite and piroplasm stage of *T. annulata*. Immune responses against the merozoite and piroplasm are considered unlikely to protect animals from the disease, since the schizont alone can cause death before the occurrence of piroplasms (Brown, 1990). On the other hand, immunity against piroplasms could play a valuable role, since it might provide a way of preventing animals becoming carriers of infection and block the transmission of the parasite and also may reduce the severity of disease (Irvin and Gill, 1981; d'Oliveira *et al.*, 1997a).

Immune serum from *T. annulata* infected animals has been shown to react with free merozoites isolated from infected erythrocytes, but not with infected erythrocytes (Ahmed *et al.*, 1988), suggesting that humoral immunity to the piroplasm stage is not effective. However, mAbs have been generated to piroplasms and these have been shown to react with *in vitro* derived merozoites but not with macroschizonts (Glascodine *et al.*, 1990). One of these mAbs, (5E1), recognised an antigen in merozoite and piroplasm stages of the parasite, which has a molecular mass of 30 kDa. This antigen was also strongly recognised by immune bovine serum and shown to exist in two forms, 30 kDa and 32 kDa, which are related to each other (Dickson and Shiels, 1993). The genes encoding the 30/32 kDa molecule (Tams1) have been isolated and characterised (Shiels *et al.*, 1994; 1995). Sequence analyses of Tams1 showed that there is significant variability within the coding sequence, particularly within the region that contains the majority of N-linked putative glycosylation sites (Katzer *et al.*, 1998a). The level of diversity may indicate selection of variable sites or amino-acid epitopes in order to evade the bovine immune system (Shiels *et al.*, 1995).

2.7.2. Cellular Immune Response to *T. annulata* infection

While the humoral response was shown to be important in *Theileria* infected cattle against the sporozoites, the cellular response has been shown to be mainly directed against the schizont-infected cells. Both the innate and adaptive immunity are believed to be responsible for protective immunity to *T. annulata* (Preston *et al.*, 1999) and cytotoxic T cells, natural killer (NK) cells, macrophages and helper T cells participate in these immune responses.

The first investigation of *T. annulata* cellular immunity was made by Preston and Brown (1981). It was shown that irradiated *T. annulata* infected cells induced proliferation of autologous peripheral mononuclear cells (PBM) whether or not the cells came from an animal which was immune. Preston *et al.* (1983) later demonstrated that recovery from primary *T. annulata* infection was accompanied by the appearance of cytotoxic cells that were not manifest in animals undergoing a fatal reaction. There were two peaks of cytotoxic cell activity following primary infection. The first peak which occurred two weeks after the contact with the parasite was bovine leukocyte antigen (BoLA = MHC class I) restricted. The second peak, observed approximately four weeks after infection, was not BoLA restricted. The same kinetics of responses occurred following challenge of immune cattle, but both peaks were BoLA restricted. It was suggested that the non-restricted activity could be due to the natural killer (NK) cells (Preston *et al.*, 1983).

Innes *et al.* (1989) further analysed cytotoxic T lymphocyte (CTL) responses in three groups of animals that were immunised with *T. annulata* sporozoites, or autologous or allogenic macroschizont infected cells. All animals developed a CTL response three weeks after the infection. However, animals infected with sporozoites or with an autologous cell line showed a severe clinical reaction and a CTL response which was not BoLA restricted. In contrast a mild clinical reaction was observed in animals infected with allogeneic cell lines and the CTL response was directed against the allogeneic MHC antigens. When animals were challenged with sporozoites, all cattle were immune and developed a secondary CTL response 10 days after challenge that was parasite specific and MHC restricted (Innes *et al.*, 1989).

In addition to cytotoxic T cells responses, cytokines and macrophage-mediated cytostasis of macroschizont-infected cells have been shown to be

components of the immune response of cattle infected with *T. annulata* (Preston and Brown, 1988; Preston *et al.*, 1993). *T. annulata* infected macrophages activate autologous T cells from naïve cattle through a combination of cytokines and contact between infected cells and T cells (Campbell *et al.*, 1995). *In vitro* studies demonstrated that the infected cells produce tumour necrosis factor alpha (TNF-α), and stimulate TNF-α synthesis in uninfected macrophages (Preston *et al.*, 1993), interferon (IFN-γ) synthesis in $CD4^+$ T cells (Campbell *et al.*, 1997) and nitric oxide (NO) in macrophages and lymphocytes (Visser *et al.*, 1995). The production of NO by macrophages is stimulated by IFN-γ. NO was assessed as a potential mediator of macrophage anti-*Theileria* activity. It was shown that NO prevents *T. annulata* sporozoites invading PBMs and also prevents trophozoite infected cells transforming into macroschizont infected cell (Visser *et al.*, 1995). Additionally, it was demonstrated that enhanced production of NO inhibits the proliferation of schizont-infected cells *in vitro,* causing macroschizonts to disappear and host cells to become apoptotic, and to kill the cells containing differentiating merozoites (Richardson *et al.*, 1998). The cellular mechanisms that control intraerythrocytic piroplasms are not known but it has been suggested that NO may also play a role to stop merozoites invading erythrocytes (Preston *et al.*, 1999).

NK cells may represent an important part in innate immunity to *T. annulata* infection. NK cells lyse schizont-infected cells and produce IFN- γ which activates uninfected macrophages to produce TNF-α and NO. NK cells are found during the healing stages of sporozoite-induced infections (Preston *et al.*, 1983) and such cells are activated by IFN- γ, interleukin-12 (IL-12) and TNF-γ which are all produced by macroschizont infected cells (Preston *et al.*, 1999).

2.8. DIAGNOSIS

2.8.1. Clinical Diagnosis

In areas where the disease is endemic the diagnosis of tropical theileriosis is based on clinical signs and macroscopic lesions (section 2.7). For laboratory confirmation, the most effective method is Giemsa's stained lymph node or liver biopsy to demonstrate the presence of macroschizonts. Thin blood smears can also be useful for clinical assessment of infection by detecting piroplasms (Sergent *et al.*,

1945; Pipano, 1994) though this may not *per se* distinguish current clinical infection from past infection and the carrier state. The advantages of blood and lymph node biopsy preparation are that the samples are easy to collect, transport and preserve, and do not require sophisticated equipment. However, smears must be of good quality and in searching for macroschizonts in smears care must be taken to differentiate between the schizont and artefacts such as normal azurophilic granules (Lawrence *et al.*, 1994). Blood smears prepared during the early stages of the disease show rare or no piroplasm infected red blood cells, a situation also observed in recovered carrier animals.

In *T. annulata* infection, schizonts are rare in the peripheral blood and their presence in blood smears indicates a poor prognosis. The number of piroplasm infected red blood cells increases progressively during the course of disease, parasitaemia reaching as high as 90%, but the severity of the disease does not necessarily correspond to the percentage piroplasm parasitaemia. After recovery, piroplasms persist for a long time but they are not always detectable by direct microscopic examination (Pipano, 1994).

In all *Theileria* species, a variety of intraerythrocytic piroplasm forms is seen, including rod, oval and comma shapes and rings (Uilenberg, 1981b). The distribution of *T. annulata* overlaps with other *Theileria* spp, notably *T. buffeli* (Purnell, 1978) and, in southern Sudan, with *T. parva* (Lomuro, 1992). The size and percentages of different forms of piroplasms sometime can help to differentiate *Theileria* species from each other where the distribution of the parasites overlaps. For example, piroplasms of *T. parva* are predominantly rod-shaped and small whereas piroplasms of *T. annulata* are predominantly round and oval, and are of moderate size. Piroplasms of *T. buffeli* are predominantly rod-shaped and often exceptionally long and associated with intra-erythrocytic structures, veil and bar. However, during the course of infection piroplasm forms can vary, rendering them unreliable for specification purposes (Uilenberg, 1981b; Norval *et al.*, 1992).

The packed cell volume (PCV) can be an adjunct procedure for diagnosis of *T. annulata* infection as the anaemia is an important symptom of the disease.

2.8.2 Antibody and Antigen Detection

The most widely used serological antibody assay for theilerial antibodies species is the indirect immunofluorescent antibody test (IFAT) using either piroplasm or cultured schizonts as antigens (Pipano and Cahana, 1969; Pipano *et al.*, 1969; Burridge, 1971; Burridge and Kimber, 1972; Burridge *et al.*, 1974). The techniques for the performance of these tests has been standardised by (FAO, 1984). The IFAT has been widely used in studies of the prevalence of *T. annulata* and for monitoring antibody responses of vaccinated animals (Pipano, 1974).

However, the IFAT has some drawbacks, notably the subjective nature of the test and a lack of specificity between *Theileria* species: *T. parva, T. annulata, T. mutans* and *T. taurotragi* all cross-reacting to some degree (Burridge *et al.*, 1974). Cross-reactions also occur between other piroplasms (*Babesia bigemina, B. bovis, Anaplasma marginale* and *A. centrale*) (FAO, 1984). Furthermore, IFAT requires expensive equipment for it to be conducted. The complement fixation test and a haemagglutination inhibition test have also been used to detect anti-theileria antibodies but their value is limited due to lack of specificity (Norval *et al.*, 1992).

Novel techniques have been developed to improve and complement the IFAT. These have primarily involved replacing the fluorescein conjugate with immunoperoxidase (Cowan, 1981; Cowan *et al.*, 1984; Campbell *et al.*, 1994a) or alkaline phosphate anti-alkaline phosphatase (APAAP) (Ahmed *et al.*, 1994). Although the reagents for both techniques are expensive, these tests have some practical advantages over IFAT as it easy and rapid to measure the antibody titres, requires only an ordinary light microscope and allows indefinite storage of material which can be examined over a number of weeks.

In the past 20 years the enzyme linked immuno-sorbent assay (ELISA) has similarly replaced the radio-immunoassay and has been increasingly used in veterinary medicine as a serological test for the diagnosis and epidemiological assessment of disease (Voller *et al.*, 1976). Wright *et al.*, (1993) outlined the development and application of these techniques in the diagnosis of infectious diseases of veterinary importance.

ELISA has several advantages over the IFAT. For example, The ELISA allows the processing of larger numbers of samples than the IFAT, it does not require

expensive equipment yet can be fully automated, making it suitable for objective screening. Thus, the technique offers the prospect of a more effective diagnostic and survey tool (Kemeny and Chantler, 1988). In addition, the potential sensitivity of ELISA is greater than IFAT. As a result, several attempts have been made to develop an ELISA to detect antibodies specific to *T. annulata* (Gray *et al.*, 1980; Kachani *et al.*, 1992; Sundar *et al.*, 1993; Reddy *et al.*, 1994; Beniwal *et al.*, 1997; Prasanth *et al.*, 1995). In these studies, crude antigen extracts obtained from piroplasms (Gray *et al.*, 1980; Sundar *et al.*, 1993), macroschizont (Reddy *et al.*, 1994; Prasanth *et al.*, 1995) or both macroschizont and piroplasm (Kachani *et al.*, 1992; Beniwal *et al.*, 1997) stages of the parasite were used. However, none of these tests were fully evaluated regarding sensitivity and specificity.

The ELISA based on crude piroplasm antigens which was developed by Kachani *et al.* (1992), based on the original method described by Gray *et al.* (1980), using sera from immunised cattle exhibited low non-specific detection by normal sera and high post-infection values. The same author also showed that the use of the piroplasm crude antigen in ELISA was more efficient than the schizont antigen. The assay has provided an opportunity for epidemiological studies of *T. annulata* infection in Morocco (Kachani *et al.*, 1994; Kachani *et al.*, 1996). However, the sensitivity and specificity of ELISA using either macroschizont or piroplasm extracts were lower than that obtained for IFAT.

Recently, stage specific recombinant antigens from the sporozoite and the merozoite/piroplasm stages of the parasite have been defined and used in ELISA systems. These include the sporozoite surface antigen, SPAG-1 (Williamson *et al.*, 1989; Hall *et al.*, 1992), merozoite rhoptry antigen, Tamr-1 (Shiels *et al.*, 1994), and the 32 kDa major merozoite and piroplasm surface antigen (Tams1) (Shiels *et al.*, 1995). ELISAs using these recombinant antigens are described in details in Chapters 5 and 6.

To date, theilerial antigen detection ELISA has only reported for *T. mutans* (Katende *et al.*, 1990). This tests offers the prospect of a more effective diagnostic and survey tool, being able detect antigens and immune complexes, allowing earlier diagnostic confirmation than antibody detection. Two mAbs that recognise different epitopes on the 32 kDa molecule of *T. mutans* piroplasms were used in a capture

ELISA, while purified 32 kDa protein was used as antigen in an antibody ELISA (Katende *et al.*, 1990). By using this test it was possible to detect both circulating antigens and antibodies successfully in serum of a reacting and/or immune cow.

There are presently two other methods to detect and identify *Theileria* infection in the host. First, the presence of macroschizonts is detected by *in vitro* isolation of peripheral blood mononuclear cells (PBM) which give any cells infected with theilerial macroschizonts an opportunity to multiply in the culture to detectable levels (Stagg *et al.*, 1974; Sharma and Brown, 1981; Kariuki *et al.*, 1995). Second, the classical or traditional method is xenodiagnosis, by which tick application to possibly infected animals and subsequent transmission of infection by the next life-cycle stage of those ticks to susceptible hosts has been used to detect the true carrier status of animals (Sergent *et al.*, 1945; Dolan, 1986). Both of these tests are laborious and time consuming and novel methods are being sought to improve and/or replace them in the detection of carrier animals.

2.8.3. Detection of parasite DNA and RNA

Recombinant DNA techniques, the use of selected parasite-specific DNA sequences cloned in plasmid vectors has allowed the presence of *Theileria* DNA in an infected animal to be detected specifically and sensitively (Allsopp *et al.*, 1993; Conrad *et al.*, 1987). This specific DNA can be detected during the prepatent, patent, convalescent and carrier periods of haemoprotozoan disease by using specific DNA probes (Figueroa and Buening, 1995). Using these techniques it has been possible to distinguish species and stocks of parasite which are not possible to differentiate solely on the basis of the morphology of the piroplasm and schizont stages (Norval *et al.*, 1992). There are several studies based on *T. parva,* the agent causing East Coast fever in East and Central Africa. By using DNA probes on restriction enzyme digested DNA from cloned *Theileria*-infected lymphoblastoid cell lines, a marked polymorphism has been shown on antigenically different *T. parva* subclones (Conrad *et al.*, 1987) and among *T. parva* stocks from different areas using probes of different specificity (Bishop *et al.*, 1994). DNA probes have also been used to detect *T. annulata* infection and have provided an opportunity to study genetic polymorphism

of parasite populations within Tunisia by detection of restriction fragment length polymorphism (RFLP) (Ben Miled *et al.*, 1994).

In order to develop a rapid and species-specific diagnostic system, 6 oligonucleotide probes from small subunit ribosomal RNA (ssu rRNA), corresponding to species-specific regions, were designed (Allsopp *et al.*, 1993). These probes were shown to provide unequivocal identification of each of the 6 species either by direct detection of parasite ssu rRNA or by hybridization to amplified parasite ssu rRNA genes. The species specific oligonucleotides from this gene was also used for development of polymerase chain reaction (PCR) methodology for *T. annulata* (de Kok *et al.*, 1993; Ilhan, 1995; Ilhan *et al.*, 1998). Later, Bishop *et al.* (1995) described the development of *Theileria* species-specific oligonucleotides derived from large subunit (LSU) ribosomal RNA sequences. These can be used for species discrimination and diagnosis of carrier animals under non-stringent conditions. They reported that the sequence of the *T. annulata* variable region was so different from that of the other four species tested that clear discrimination was obtained among the five species using oligonucleotide probes against PCR amplified LSU RNA gene sequences. Moreover, the sensitivity of DNA detection by the LSU oligonucleotide was comparable to the oligonucleotide derived from *Theileria* ssu rRNA gene sequences. This result has suggested that the multiple oligonucleotides derived from LSU RNA genes will be useful for application in detection of carrier state and identification of infected ticks.

With the advent of the polymerase chain reaction (PCR) technique (Saiki *et al.*, 1988a) positive detection and identification of very small numbers of parasites in the vertebrate host and tick vector has become possible. Bishop *et al.* (1992) has developed a PCR method to detect carrier state of cattle infected with *T. parva* by amplification of parasite DNA by using oligonucleotide primers for *T. parva* specific a repetitive DNA sequence. The same primers were also used for identification and quantification of *T. parva* in the vector tick *Rhipicephalus appendiculatus* (Watt *et al.*, 1997) and showed that the test sensitively detected the infection in ethanol preserved, partially fed or unfed ticks.

In recent years, several different PCR methodologies have been reported for the detection of *T. annulata* in either vector tick (de Kok *et al.*, 1993; d'Oliveira *et*

al., 1997b) or bovine host (d'Oliveira *et al.*, 1995; Ilhan *et al.*, 1998; Shayan *et al.*, 1998; Gubbels *et al.*, 1999b; Leemans *et al.*, 1999a; MartinSanchez *et al.*, 1999). These PCR methodologies were mainly based on primers from either ssu rRNA (de Kok *et al.*, 1993; Ilhan *et al.*, 1998; Gubbels *et al.*, 1999b) or the gene encoding the major surface protein (Tams1) (d'Oliveira *et al.*, 1995; Leemans *et al.*, 1999a; MartinSanchez *et al.*, 1999). The sensitivity of these tests differed widely. For example, the PCR developed by d'Oliveira *et al.*, (1995) for specific amplification of *T. annulata* DNA from blood samples was able to amplify DNA at 0.00005% parasitaemia. Another PCR developed using primers from ssu rRNA was able to amplify *T. annulata* DNA at 0.0000025 % parasitaemia (20x more sensitive) (Ilhan *et al.*, 1998). However, the common feature of these tests was to be more sensitive and specific than conventional tests used for detection of the parasite, i.e. microscopic examination of Giemsa's stained thin blood smears and methyl green pyronin or feulgen staining of tick salivary glands (Walker *et al.*, 1979).

Recently, a reverse line blot (RLB) technique was developed for the identification of *Theileria* and *Babesia* simultaneously where the mixed infections occur (Gubbels *et al.*, 1999b). The essence of this technique is the hybridisation of PCR products to specific probes immobilised on a membrane in order to identify differences in the amplified sequence. The sensitivity of the test was determined at 0.000001% parasitaemia and it specifically detected six bovine *Theileria* spp (*T. annulata, T. parva, T. mutans, T. taurotragi, T. velifera* and *T. orientalis)* and three *Babesia* spp (*B. bovis, B. divergens* and *B. bigemina*) tested. This assay gives the opportunity to collect information concerning the epidemiology of pathogenic parasites simultaneously and also non-pathogenic parasites that shows cross reactivity with pathogenic ones. Additionally, the membrane with the covalently linked species specific oligonucleotides can be used at least 20 times which reduces the costs for screening animals.

2.9. CONTROL OF TROPICAL THEILERIOSIS

Four main approaches to control tropical theileriosis are available: vector control, chemotherapy, breeding cattle resistant to theileriosis and vaccination. These

measures in recent years have been reviewed by a number of authors including Brown (1990), Dolan (1989) and Tait and Hall (1990).

2.9.1. Vector Control

Farm management and control of the vector ticks may play significant roles in reducing the impact of tropical theileriosis in endemic areas. The main aims at control of transmission of the disease is either to reduce the contact between the animals and vector ticks to such an extent that transmission does not occur or deliberately expose to cattle at a time when they are most likely to resist the impact of the disease resulting in the development of endemic stability (Brown, 1990). Control of the tick involves the use of acaricide application to the cattle and cowsheds which ticks may reside in. Improvement in the construction of animal accommodation to remove sites in which ticks can hide makes the acaricide use more effective.

The use of acaricide for tick control is most commonly implemented by dipping animals in tanks containing a solution of the acaricides. This method is more effective than spraying in achieving satisfactory coverage of cattle by the acaricides (Norval *et al.*, 1992). Other methods of applying acaricides include spot or pour-on application, slow release acaricide boluses and acaricide-impregnated ear tags (Kocan, 1995). The use of the chemotherapeutic agent ivermectin has been shown to be effective for tick control by acting on nervous system receptors of the ticks (Wilson, 1993).

The use of acaricides in tick control has been reviewed (FAO, 1984), but there are several problems with their use in the control of tick-borne diseases (Tait and Hall, 1990): (i) The need for highly organised dipping programmes to ensure all cattle are treated. (ii) Long term application can result in the development of acaricide resistance in the ticks. (iii) Continual use of acaricides interferes with the development of endemic stability that can exist in indigenous breeds when calves are exposed to naturally transmitted tick borne diseases. These cattle populations are at risk from disease outbreaks when acaricide treatment is interrupted for any reason. Additional disadvantages of using acaricides are their increasing cost, toxic residues in animal products and environmental pollution (Norval *et al.*, 1992).

The use of vaccines against ticks would be a potential measure to integrate into control programmes. The immunology of tick-host interactions has recently been reviewed (Willadsen and Jongejan, 1999). There is evidence that it is possible to vaccinate livestock against ticks using gut extract. In recent years, commercial vaccines have been produced against the one-host tick *Boophilus microplus*, the vector of both anaplasmosis and babesiosis of cattle. The vaccines rely on an artificially induced immune response to internal or 'concealed' antigens, for example Bm86, in the gut or other tissue and this has been shown to be effective in field trials in Australia (Willadsen *et al.*, 1995), Cuba (Rodriguez *et al.*, 1995b) and Brazil (Rodriguez *et al.*, 1995a). Vaccination of cattle leads to reduction in the number of engorging female ticks and their weight and fecundity. Bm86, or its equivalent, is present in other tick species. In *H. anatolicum anatolicum,* a 100 amino acid sequence has 76% similarity to the *B. microplus* Bm86, but its significance as a vaccine is not fully known (Willadsen and Jongejan, 1999).

2.9.2. Chemotherapy

In areas where a vaccine against tropical theileriosis is not yet widely available disease control is based on acaricide use and chemotherapy. Early studies reported a wide range of drugs, including antibacterial and antiprotozoal drugs that have been tested, but none showed specific antitheilerial action (Neitz, 1957). Tetracycline compounds, which have also been used (Neitz, 1957), but have little effect once the clinical signs of tropical theileriosis are apparent (Hashemi-Fesharki, 1974).

In the last 15-20 years novel, effective and specific drugs have been developed to use against theileriosis. The first of these was menoctone (McHardy *et al.*, 1976), an anti-malarial naphthoquinone, which was highly effective but too expensive. Another theilerial drug, halofuginone was identified as effective against *T. annulata* infection (Schein and Voigt, 1979), but it is toxic for the animals at near therapeutic doses. Then, a second naphthoquinone, parvaquone, was found to be effective against *T. annulata* (Gill *et al.*, 1981). Parvaquone was followed by buparvaquone which was several times more effective than parvaquone (McHardy *et al.*, 1985). Buparvaquone is now marketed widely as Butalex® (Shering). These

drugs are safe and very effective against the disease but are still expensive (Brown, 1990). In addition, if treatment is to be effective it needs to be administered at an early stage of clinical disease. Early diagnosis and an efficient veterinary service are thus of great importance.

2.9.3. Resistance of Cattle to Tropical Theileriosis

In the long term, the use of breeds resistant to both theileriosis and the tick vector will play a major role in the pragmatic control of theileriosis (Brown, 1990). This may indeed prove to be the ultimate sustainable solution to this disease. It has been stated in a review of the subject (Campbell, 1978) that *Bos indicus* cross cattle may carry 10% to 40% fewer ticks than *Bos taurus*. The practical advantages of using *Bos indicus* and *Bos indicus* cross cattle in northern Australia are widely accepted due to the recognised tick resistance, improved weight gains, heat tolerance and more efficient utilisation of feed. In a recent study in Burundi host resistance to ticks on cross-bred cattle between higher producing exotic breeds and indigenous Ankole cattle showed that the Ankole/Sahiwal cross-bred exhibited marked host resistance to ticks, especially *Rhipicephalus appendiculatus* (Moran *et al.*, 1996).

In the contexts of studies have shown that there is variation between animals in their response to a low dose of parasite within Friesian and Ayrshire (*Bos taurus*) calves examined, some being more resistant than others (Preston *et al.*, 1992). The current problem with the use of genetically resistant cattle is the difficulty in simultaneously selecting and breeding for resistance and productivity. Therefore, if the genes determining resistance could be identified, introducing these into highly productive cattle is a possibility (Spooner and Brown, 1991). In the face of a committed drive, in Turkey and elsewhere towards selective breeding of milk producing cattle based on Friesian or Holstein genotypes, some interim means of disease control will be necessary until such resistant cattle can be generated.

In the meantime until both productive and genetically resistant cattle have been identified or constructed, alternative means of control need to be used. To date the most effective and practical method is by immunisation using an attenuated culture vaccine.

2.9.4. Immunisation

2.10.4.1. Live vaccines- attenuated cell lines

The production and the use of schizont tissue culture vaccines have been described by many authors including Pipano (1989b), Norval *et al.* (1992), Pipano (1995) and Boulter and Hall (1999).

Vaccination with blood from cattle infected with field isolates of *T. annulata* of naturally low virulence was used by the French investigators working in the 1920s and 30s in Algeria and this method applied widely where the disease exerted an important constraint. The result of these studies is summarised by Sergent *et al.*, (1945).

Sergent *et al.* (1932) tested several isolates passaged mechanically between cattle with the aim of detecting a naturally avirulent strain. They selected the Kouba strain on this basis. Continuous mechanical passage of *T. annulata* Kouba strain in cattle resulted in the loss of ability of the parasite to produce piroplasms, i.e. infection in ticks but retain immunogenicity (Sergent *et al.*, 1945). This strain was used for vaccination from the 52nd to the 220th cattle passage, a period of over 10 years by inoculating 5-10 ml of blood from a donor calf. A mortality of 3.2% was caused by this strain among the calves used for passage in the laboratory. In Israel, Adler and Ellenbogen (1934) were unable to isolate an indigenous *T. annulata* strain of low virulence and they used the Algerian (Kouba and Brunette) strains of low virulence. These provided poor protection against the local virulent isolates. To overcome this problem, a two step vaccination procedure was adopted: first inoculating a mild exotic strain and then, 2 months later, inoculating a local virulent strain in order to reinforce the partial immunity induced by the first inoculation.

The successful cultivation of *T. annulata* schizonts *in vitro* by Tsur (1945) led to the development of a culture-derived vaccine against tropical theileriosis. This was consolidated later when Tsur *et al.* (1964) achieved the mass propagation of the parasite in tissue culture. Prolonged cultivation of a *T. annulata* infected cell line results in attenuation of the virulence of the parasite (Pipano and Tsur, 1966). Complete attenuation is achieved when the cultured schizonts no longer cause parasitaemia or clinical and parasitological symptoms in inoculated cattle (Pipano, 1977). Despite this the antibody response against macroschizont antigens, as

measured by IFAT, remains similar to that obtained after infection with virulent schizonts (Pipano, 1989b). The cell culture vaccine protects most breeds of cattle against homologous challenge and usually against heterologous challenge (Pipano, 1981).

T. annulata cell cultures were used in several countries following further development by Pipano (1981), Hashemi-Fesharki (1988), Onar (1989), Ouhelli (1991), Zablotsky (1991), Singh (1991). In many other countries, studies are under way to produce their cell line vaccines such as Iraq (Khdier and Latif, 1991), Tunisia (Darghouth *et al.*, 1996a), Morocco (Ouhelli *et al.*, 1997) and Spain (Viseras *et al.*, 1997; Viseras *et al.*, 1998). The number of cells per vaccine dose varies between 10^4 - 10^7. The duration of immunity after cell line immunisation in the absence of reinfection has not been fully investigated. However, it was reported that it ranges between 1 year (Pipano, 1977) and 3.5 years (Zablotsky, 1991). Recently, Ouhelli *et al.* (1994) showed that some animals immunised with 10^4 *T. annulata* infected cells were not protected when challenged with sporozoites after seven months.

Following inoculation with *T. annulata* schizont infected cells, there is a transfer of parasites from donor to recipient cells. This was demonstrated by having donor and recipient of different sexes and karyotyping parasitised cells before and after transfer (Brown *et al.*, 1978a). The mechanisms by which the parasite transfers are not known. However, a process involving opsonisation was suggested (Forsyth *et al.*, 1997). Schizont infected cells express CD11b, the membrane receptor for C3bi, which is a component of the complement cascade and an opsonin. It is suggested that schizonts freed from the donor cells by a rapid host response to inoculation with allogenic cells and free schizonts would be obsonized by complement and/or antibody, and then linked to cells bearing the C3bi receptors, i.e. macrophages. Attachment of the parasite-complement complex would facilitate phagocytosis of the parasite by the cell.

There are no indications that the time needed for attenuation is proportional to initial virulence of schizonts. Different isolates however require different cultivation times in order to become completely attenuated (Hooshmand-Rad, 1973; Pipano, 1989b). Complete attenuation is accompanied by the loss of ability to produce merozoites thus piroplasms (Pipano, 1977). The mechanism of attenuation

or reduction of virulence of the parasite after prolonged cultivation is not fully understood. It may be simply a clonal selection over a period of time (Brown, 1990). This reduction of virulence of *T. annulata* infected cell lines would appear to be a selection of a particular genotype (Sutherland *et al.*, 1993). Studies have been carried out to attempt to define the markers for attenuation. Immunofluorescence studies using a mAb, EU106, which specifically recognizes virulent *T. annulata* cell lines revealed alterations in antibody reactivity following *in vitro* culture (Sutherland *et al.*, 1996). The antigen recognised by the mAb EU106 is stage specific and is also expressed on the surface of host cells infected with virulent cell lines (Preston *et al.*, 1998) and its expression diminishes upon attenuation. A number of genes have been identified that are either upregulated or downregulated on attenuation (Somerville *et al.*, 1998b). Additionally, changes were observed in the host matrix metalloproteinase profile of some of the attenuated *T. annulata* infected cell lines (Baylis *et al.*, 1992; Baylis *et al.*, 1995; Adamson and Hall, 1997; Somerville *et al.*, 1998b). Additionally, it was demonstrated that the attenuation could be related to the degree of T-cell stimulatory ability of infected cells and the levels of pro-inflammatory cytokines they produce (Brown, 1997b; Brown *et al.*, 1998b). The low levels of these two factor produced by the clonal cell lines induced less severe reaction than those that the cell lines produced at high levels.

2.10.4.2. Inactivated vaccine- Subunit vaccine development

The application of an attenuated vaccine has a number of major drawbacks. These include a short shelf life, approximately 1 week at 20°C or 1 month at 4°C, the need for frozen transfer between laboratories and sites of immunisation, and the risk of infection of immunised cattle with other pathogens. Preparation of a vaccine is labour intensive and batch control is expensive (Brown, 1990; Tait and Hall, 1990). Thus, while the attenuated cell culture vaccine is very effective, these drawbacks have necessitated look for inactivated vaccine.

A number of potential antigens have been characterised including the sporozoite specific antigens, SPAG-1 of *T. annulata* (Hall *et al.*, 1992), and p67 from *T. parva* (Nene *et al.*, 1992), and the 30kDa major merozoite surface antigen of *T. annulata*, Tams1 (Glascodine *et al.*, 1990).

The SPAG-1 antigen was identified by a monoclonal antibody which inhibited sporozoite penetration of bovine peripheral blood mononuclear cells *in vitro* (Williamson *et al.*, 1989). The SPAG-1 gene was isolated and sequenced (Hall *et al.*, 1992), and has recently been demonstrated to have cross reactive epitopes with the p67 *T. parva* sporozoite antigen (Knight *et al.*, 1996). The neutralising determinant on SPAG-1, recognised by the antibody which inhibited sporozoite penetration of host cells, was mapped to 16 amino acids which implicated the C-terminus of the protein as an immunologically relevant region that can be recognised by the bovine immune response (Boulter *et al.*, 1994). Several vaccination trials have been performed using SPAG-1 recombinant antigens with various outcomes (Williamson, 1988; Knight, 1993; Boulter *et al.*, 1994; 1995; 1998; 1999). Some protection against the disease was achieved in these trials, as assessed by a reduction in the severity of the disease. Immunised animals had a significantly longer prepatent period (length of time until schizonts were seen in the lymph node). The clinical symptoms of the disease were delayed, macroschizont parasitosis was reduced and there was a significant reduction in the level of macroschizonts and piroplasms. Efficacy of the immunisation with SPAG-1 differed according to the different delivery systems used (Boulter *et al.*, 1999). SPAG-1 administered with SKBA adjuvant RWL® gave a better protection against challenge by *T. annulata* sporozoites than when combined with saponin, Freund's or ISCOMs.

The *T. parva* sporozoite antigen p67, a gene related to SPAG-1, has been extensively used in vaccination trials. The p67 antigen has been expressed in a number of systems including *E. coli* (Musoke *et al.*, 1992; Nene *et al.*, 1996), baculovirus (Nene *et al.*, 1995), vaccinia (Honda *et al.*, 1998) and *Salmonella dublin* (Gentschev *et al.*, 1998; Heussler *et al.*, 1998). Immunisation with five monthly doses of recombinant p67 induced neutralising antibodies and protected six of the nine animals challenged with *T. parva* sporozoites (Musoke *et al.*, 1992). In another vaccination trial 7 out of 12 calves were protected against homologous challenge and 6 out of 11 cattle against heterologous challenge (Nene *et al.*, 1996). In order to obtain native p67 protein a baculovirus expression system was used. Immunisation with a baculovirus-derived p67 resulted in protection in four of six Boran cattle (Nene *et al.*, 1995). Recently, it was reported that 70% protection was obtained in

trials using a total of 86 cattle (Morrison and McKeever, 1998). In all these vaccination trials, multiple doses (3 to 5 doses) of vaccine were used and this is not a practical delivery strategy. In order to reduce the number of vaccination doses two live delivery systems have been evaluated, *Salmonella* and vaccinia. The p67 expressed in a secreted form by *S. dublin* produced better protection than the internally located antigen (Gentschev *et al.*, 1998). Honda *et al.* (1998) evaluated vaccinia virus as a delivery system for p67 recombinant antigen. The p67 vaccinia construct given alone to seven animals did not produce any protection. However, 5 of 7 animals receiving a combined p67-IL-2 vaccinia construct were immune to the sporozoite challenge. To date the best protection level achieved by p67 recombinant sporozoite antigen is 70%. To achieve a total protection probably requires a sub-unit vaccine containing antigens from all parasite life-cycle stages.

The merozoite stage is, like the sporozoite, invasive and also a potential target for a protective immune response. The most abundant and immunodominant antigen on the surface of the merozoite of *T. annulata* is a 30kDa surface antigen which is conserved throughout all species of *Theileria* analysed to date (Glascodine *et al.*, 1990; Dickson and Shiels, 1993). Recently, allelic forms of Tams1 (Tams1-1 and Tams1-2) which encode the 30- 32kDa major merozoite antigens of *T. annulata* respectively, were expressed in a *Salmonella typhimurium* aroA vaccine strain and *Esherichia* coli for inclusion in a sub-unit vaccine (d'Oliveira *et al.*, 1996). Preliminary immunisation trials with the recombinant antigens indicate that there may be some protection against challenge (d'Oliveira *et al.*, 1997a).

2.10. TROPICAL THEILERIOSIS IN TURKEY

Tropical theileriosis is the most important cattle disease in Turkey and occurs throughout the country (Mimioglu *et al.*, 1971; Mimioglu *et al.*, 1972). The presence of tropical theileriosis was reported for the first time early this century by Celebi (1912; 1925) together with the presence of vector ticks of *T. annulata*. These reports were followed by many others from every climatic region of Turkey, except the mountainous areas where climatic conditions are not favourable for the vector tick (Samuel and Raif, 1930; Tüzdil, 1954; Kurtpinar, 1954; Göksu, 1959; Mimioglu *et al.*, 1971; Tüzer, 1982; Dumanli and Özer, 1987; Cakmak and Öz, 1993; Alp, 1996).

T. annulata constitutes a major threat to European and crossbred cattle in particular (Mimioglu *et al.*, 1971). The mortality rate in such cattle was recorded as 53% (Güler, 1985) with figures of up to 100% and up to 43% in indigenous breeds (Göksu, 1959; Mimioglu *et al.*, 1971; 1972).

In Turkey, the most important *Hyalomma* spp. is three-host tick *H. anatolicum anatolicum* and the two-host tick *H. detritum* (Dumanli, 1989). *H. excavatum* and *H. marginatum* are also frequently recorded (Kurtpinar, 1954; Hoffman *et al.*, 1971; Karaer, 1981; Sayin *et al.*, 1991). Cases of tropical theileriosis occur from May until September (this may change according to climatic zone extending to between April and November), with the highest number occurring in June and July, in parallel with the increase in numbers of adult *Hyalomma* ticks (Göksu, 1959; Dumanli and Özer, 1987).

The livestock industry plays a vital in the agricultural economy of Turkey and provides 35% of the value of agricultural production. There are approximately 16 million cattle in Turkey of which 85% are local breed, 13% cross breeds and 2% European breeds. Despite the high number of animals, the production level per animal is not satisfactory. One of the main features of industry is the introduction of European breeds (mainly Holstein-Friesian and Brown Swiss dairy cattle) into areas with high agricultural potential to replace the local breeds. Theileriosis becomes important when such fully susceptible exotic cattle are introduced to an endemic area. Susceptibility to the disease was shown to be age dependent as clinical cases were reported mainly in adult cattle (Göksu, 1959; Tüzer, 1982), possibly because more ticks are found on adult cattle than on calves. Nevertheless, theileriosis cases are reported in calves under one month old (Dumanli and Özer, 1987).

The distribution of another species of Theileria '*T. mutans*' in Turkey has been reported as being widespread on the basis of microscopic examination (Mimioglu *et al.*, 1971; Güler, 1978; Tüzer, 1982). It is now accepted that *T. mutans* is mainly an African parasite occurring south of the Sahara (Norval *et al.*, 1992). Uilenberg *et al.* (1974) and Young *et al.* (1978) proved that *T. mutans* is transmitted by ticks of *Amblyomma* species. There are no records of the presence of this tick in Turkey (Kurtpinar, 1954; Merdivenci, 1969). However, *T. buffeli/T. orientalis*, which is similar in appearance to *T. mutans* and of similar low pathogenicity is transmitted

by *Haemaphysalis* spp. (Uilenberg, 1981b), which are widespread in Turkey (Kurtpinar, 1954; Merdivenci, 1969). These observations lead to the conclusion that the second theilerial species of cattle observed in Turkey may in fact be *T. buffeli/T. orientalis*.

The control of theileriosis in Turkey has been carried out by vector control, treatment and the use of an attenuated macroschizont-infected cell culture vaccine. Tick control by acaricides is not always because excessive and frequent use leads to acaricide resistant tick strains. Also the accumulation of drug residues in milk and meat is unacceptable (Onar, 1989). Acaricidal control is only applicable in large-scale animal holdings. For the small holder farms vector control is either very poor, or not carried out effectively at all. Disease control was limited in Turkey until buparvaquone (Butalex) was introduced into the Turkish market and used effectively in the treatment of tropical theileriosis in the field (Ünsüren and Kurtdede, 1988).

In recent years the main control method for theileriosis has been vaccination using an attenuated *T. annulata* macroschizont-infected cell line. This first became available for use in 1982 (Ozkoc and Pipano, 1981; Onar, 1989). Preliminary studies for preparation and application of this vaccine started with the isolation of stocks from different regions of Turkey (Schein *et al.*, 1975; Özkoc *et al.*, 1978) in co-operation with Israeli researchers (Pipano, 1989a). This isolation was followed by pathogenicity tests of stocks isolated from different regions of Turkey, the Ankara, Kirikkale, Hatay, Diyarbakir and Gebze 'strains'. Four out of these strains (excepting Kirikkale) were found to be highly virulent (Özkoc and Onar, 1980; Vural *et al.*, 1978). The strains of *T. annulata* isolated from different regions of Turkey have been cultivated and passaged *in vitro* over various time periods for use in vaccination trials. The strain designated 'Ankara' has been used for vaccine production and passaged until complete attenuation was obtained at passage level 250 (Ozkoc and Pipano, 1981). After this passage level, the cell line was used experimentally in the laboratory (Ozkoc and Pipano, 1981) and field tested in different regions of Turkey, (Onar, 1989). The conclusion was that this attenuated cell culture 'vaccine' was effective enough to be produced for widespread use throughout Turkey. Vaccine production was started with 9,500 doses (10^7 cell per dose), in 1982 and reached 150,000 doses of vaccine in 1988. In subsequent years the vaccine has been

produced at approximately this level (Onar, 1989). The application of the cell line vaccine in endemic areas was interrupted from 1992-1995 due to the contamination of the vaccine with other pathogenic organisms (Sayin pers. comm.).

With the increasing numbers of European dairy cattle being imported into Turkey and cross-breeding to improve productivity of indigenous cattle, 150,000 doses of vaccine scarcely meets the demand. There is thus, currently an initiative to improve the viability of the vaccine and to reduce the cell dose required. Efficient cost recovery will also lead to sustainable production and delivery. In the meantime effective targeting and prioritising of vaccine delivery is important. This depends on the provision of evidence that the vaccine works and is the current control method of choice in Turkey. This study aims to develop tools to enable these points to be dealt with, namely, to provide appropriate tests to study the epidemiology of the disease and to help in determining the ability of vaccinated cattle to withstand challenge in the field by unequivocally showing which animals have undergone a field challenge.

CHAPTER THREE

IMMUNITY OF CALVES TO THEILERIA ANNULATA FOLLOWING IMMUNISATION WITH SPOROZOITES AND MACROSCHIZONT INFECTED CELL LINES

3.1. INTRODUCTION

Attenuated macroschizont infected cell lines have been used in several countries to control tropical theileriosis and to protect exotic and improved cattle (Brown, 1990). In Turkey, tropical theileriosis represents a major threat to improved dairy and beef breeds. At present control of the disease is based on the use of acaricides against the vector tick and on a live attenuated macroschizont vaccine. However, only 150,000 doses of vaccine are produced each year and this is not sufficient to meet the demand (Onar, 1989).

Successful deployment of a vaccine depends on the production of the vaccine and on the design of the vaccination programme. This requires knowledge of the vaccine's characteristics and the epidemiology of the target parasite or pathogen. There are a number of factors that must be determined in the design of a successful vaccination programme. These factors include: the proportion of hosts that must be vaccinated, the age at which hosts should first be vaccinated and at what interval, if at all, hosts should be re-vaccinated (Woolhouse and Bundy, 1997).

Regarding vaccine characteristics, experimental studies are needed not just to determine vaccine take and the degree of protection afforded but also how this protection wanes through time and how each of these might vary with host age and vaccination history (Woolhouse *et al.*, 1997). There is little information about the duration of immunity engendered by vaccination against tropical theileriosis using attenuated macroschizont infected cell lines. Available information suggests that the length of protection ranges from less than 1 year to 3.5 years (Pipano, 1989b). However, the requirement for the revaccination is not known.

The disease is distributed unevenly between the vector ticks and the susceptible host populations. Understanding parasite population dynamics and epidemiology helps in the design of immunisation programmes. It is particularly

important that vaccination is given to the hosts most at risk of infection. Good diagnostic tests are required to identify such populations. These diagnostic tests also provide tools for evaluating vaccine efficacy in the field. Current diagnostic methods for tropical theileriosis do not distinguish between animals that have only been vaccinated and those that have been naturally infected or vaccinated/challenged. This makes it impossible to determine the true prevalence of *T. annulata* in areas where vaccination is practised. It is also not easy to study large-scale impact of vaccination by using current diagnostic tests. Therefore, it is necessary to develop new diagnostic methods to distinguish vaccinated animals from naturally infected animals, and so obtain accurate epidemiological data.

The present study was carried out to extend a pilot study initiated by Ilhan (1995) with the following aims: (i) provide materials for development of an ELISA to distinguish vaccinated animals from naturally infected animals, (ii) evaluate, within the time available, the duration of immune responses after infection (and vaccination) of cattle with different life-cycle stages of the parasite, (iii) examine the stage-specific immunity against *T. annulata* in cattle infected with different life-cycle stages of the parasite, (iv) examine the relationship between the carrier state and immunity of the cattle to challenge. The data obtained in the pilot study (Ilhan, 1995) are presented along with results obtained in the current study for the purpose of clarity.

The experimental design used in the current study was similar to that described previously (Ilhan, 1995). Calves were infected in different ways to simulate the field situation, i.e. natural infection and vaccination, in the laboratory. Three main groups of animals were infected with *T. annulata*. The first group received sporozoites to simulate natural tick infection; the second group was infected with a low passage cell line to simulate calves vaccinated with a non-attenuated cell line which gives 'carrier' state; and the third group was infected with high passage attenuated cell lines to simulate vaccination with a fully attenuated non-piroplasm producing vaccine cell line. The calves were then challenged 1 or 7 months after primary infection with a heterologous sporozoite stabilate.

3.2. MATERIALS AND METHODS

3.2.1. Experimental Animals

Twenty-five Friesian or Ayrshire castrated calves (2-3 month old, male) were obtained from farms near Edinburgh. After arrival at the Centre for Tropical Veterinary Medicine (CTVM) calves were kept in quarantine until confirmed negative for *Salmonella* infection by faecal culture. Animals were kept indoors and fed twice a day with commercial calf pellets and *ad libitum* with hay and water.

3.2.2. Parasite Material

A Turkish stock of *T. annulata* Ankara was used to infect or immunise calves either in the form of cryopreserved, sporozoite stabilates or as macroschizont infected cell cultures, *viz. T. annulata* Ankara low passage (p5) (Schein *et al.*, 1975), *or T. annulata* Ankara/Pendik high passage or 'vaccine' (p317) (Ozkoc and Pipano, 1981). Cryopreserved sporozoite stabilate of a Moroccan isolate, *T. annulata* Gharb (Ouhelli, 1985) was used to challenge the calves.

T. annulata Ankara was isolated near Ankara and brought to Berlin (Schein *et al.*, 1975). A heavily infected batch of laboratory *Hyalomma anatolicum excavatum* ticks was sent to the Centre for Tropical Veterinary Medicine (CTVM). The stock was maintained at the CTVM by alternate tick/cattle passage. Sporozoites were harvested and used to infect peripheral blood mononuclear cells (PBM) in culture (Brown, 1983). *T. annulata* Ankara low passage cell line (p3) was established by *in vitro* infection of PBM of calf 279.

T. annulata Ankara isolated by Schein *et al.* (1975) was sent to Pendik Veterinary Research Institute, near Istanbul and a cell line established *ex vivo* by establishment of macroschizont infected cells. This cell line was attenuated by prolonged *in vitro* passage and designated as *T. annulata* Ankara/Pendik. It is currently used as a vaccine in Turkey (Ozkoc and Pipano, 1981).

Ground-up tick supernatant (GUTS) stabilates containing sporozoites were stored at –180°C in liquid nitrogen at CTVM. The stabilates were prepared according

to established procedures (Brown, 1983) and resuscitated for use as described in section 3.2.3.2.

3.2.3. *In vivo* Experimental Design

3.2.3.1. Groups of experimental calves

Twenty-five calves were divided into four main groups numbered 1 to 4. Each of these groups was challenged along with four control calves constituting the fourth group at the same time. Table 3.1 shows details of the experimental Groups.

Table 3.1. Experimental design of calves for infection and immunisation

Group No	Calf No	Inoculation of *T. annulata*	Infection to challenge interval	Challenge
1	21A, 23B, 48C, 897A	Ankara sporozoite stabilate 54 at 0.2 tick equivalent	7 months	*T. annulata* Gharb sporozoite stabilate 63 and 67 at 1 tick equivalent
2A	22A, 24B, 17, 32C, 892A	Ankara cell line 279 p5	7 months	
2B	302A, 309A, 15, 535A		35 days	
3A	26A, 32A, 54C, 891A	Ankara/Pendik cell line p317	7 months	
3B	303A, 308A, 528, 532		35 days	
4 (Control)	203A, 301A, 13, 508	-	-	

3.2.3.2. Infection of calves

a) Sporozoite stabilate

T. annulata Ankara GUTS stabilate 54 was used to infect calves in Group 1. The sporozoite stabilate, which had been cryopreserved in 7.5% glycerol at 2 tick equivalent (t.e.)/ml, was resuscitated by thawing rapidly at 37°C and then allowed to equilibrate at room temperature for 15-20 minutes, prior to ten fold dilution with Minimum Essential Medium (MEM) (Gibco) containing 3.5% bovine plasma albumin (BPA) (Sigma) with 7.5% glycerol (Analar, BDH). The diluted stabilate was allowed to equilibrate at room temperature for a further 10 minutes. Then 1 ml of the diluted sporozoite suspension containing 0.2 t.e. was inoculated subcutaneously in front of the right shoulder of each calf in Group 1.

b) Schizont infected cells

On the day of infection, cells from 48 hour *T. annulata* schizont -infected mononuclear cell cultures in the logarithmic phase of growth were counted and their viability determined. Calves in Groups 2 and 3 were inoculated subcutaneously in front of the right shoulder with a dose of 2 x 10^6 viable schizont infected cells in tissue culture medium.

3.2.3.3. Challenge of calves

T. annulata Gharb sporozoite stabilates 63 for the first half of experiment (Ilhan, 1995) and stabilate 63 and 67, which were pooled together, in the current experiment were used to challenge all the calves (Groups 1-4) at a dose of 1 t.e. as described in section 3.2.3.2. One ml, equivalent to 1 t.e. was inoculated subcutaneously over the front of the left shoulder of each calf. The calves in the control Group 4 were inoculated last. The sporozoite stabilate was inoculated into the animals within 30 minutes after thawing.

3.2.3.4. Monitoring of infection and challenge reactions

Calves were routinely monitored from the day of infection (day 0) or challenge for 35 day as follows:

a) Clinical

Rectal temperatures were recorded daily. Animals with a temperature above 39.5°C were considered to be febrile. During the course of infection the clinical signs of the disease including pyrexia, depression, inappetance, weakness, lachrymation, petechial haemorrhages, lymph node enlargement, especially on the side of infection and anaemia were assessed.

b) Haematological

Jugular blood was taken three times a week into vacutainer tubes (Becton Dickinson) containing the di-sodium salt of ethylenediaminetetra-acetic acid (EDTA) for preparation of blood smears and haematological examination. Total white blood cell (WBC) counts and red blood cell (RBC) counts were measured using a Coulter Counter (ZM, Coulter Electronics Ltd). The packed cell volume (PCV) was determined using a haematocrit centrifuge (Heraeus).

c) Parasitological

Lymph nodes

The presence of schizonts, (macroschizonts/microschizonts) and hyperplastic cells in the draining lymph node, right for first infection, left for challenge were monitored by examination of Giemsa's stained needle biopsy smears which were prepared three times a week starting on day 5 post-inoculation of sporozoite stabilate or day 9 post-inoculation of cell lines. The biopsy sampling was continued until schizonts were no longer detected in two consecutive biopsies or until day 28 post-inoculation. Lymph node biopsy smears were air dried, fixed in absolute methanol (BDH) for 2-3 minutes and stained in 5% Giemsa's stain (Merck) in Giemsa's stain buffer (pH 7.2, BDH) for 40 minutes.

The smears were rinsed in Giemsa's stain buffer, air-dried and examined under oil immersion at 500 x and 1000 x magnification using an Ortholux II microscope (Leitz, Wetzlar, Germany). The degree of schizont parasitosis was evaluated as, + (less than 1% of cells containing schizonts), ++ (1-5 % of cells containing schizonts), +++ (more than 5% of cells containing schizonts).

Blood smears

The presence and level of piroplasm parasitaemia were monitored by examining thin blood smears taken three times a week during the first 35 days of infection and challenge and daily during the clinical reaction. Blood smears were prepared at 28-day intervals following infection. The blood smears were stained with Giemsa's stain as described above. The degree of parasitaemia was recorded as the percentage of infected red blood cells determined after counting 1000 cells. If the parasitaemia was less than 0.1%, the number of piroplasms per 100 fields at 1000 x magnification was assessed. The smear was recorded as negative if 200 fields (approximately 100,000 RBC) were examined and no piroplasms were observed.

d) Cell culture

Jugular blood was taken into vacutainer tubes containing lithium-heparin (Becton-Dickinson) in order to isolate *T. annulata* infected cells from the calves during patent infection and following recovery until challenge. Blood samples were taken on days 9, 12, 14, 16, 28 and then 28-day intervals until challenge.

e) PCR

Blood was taken into EDTA containing vacutainer tubes for evaluation by PCR. Blood samples were taken on days 0, 2, 5, 7, 9, 12, 14, 28 and then 28-day intervals until challenge. Following challenge, samples were taken on days 7 and 14.

f) Serology

Blood was taken into plain vacutainer tubes (Becton Dickinson) for IFAT and ELISA at the following times: two hours before infection; days 7, 14, 21, 28 and then 28-day intervals until challenge; one day before challenge; days 7, 14, 28 and 42 following challenge. The serum samples were aliquoted and stored at –20°C until use. Serum samples were tested in ELISA and IFAT as described in Chapter 6.

3.2.3.5. Treatment

During the primary infection and challenge animals which underwent severe reaction were treated with buparvaquone (Butalex, Shering) once or more at a dose of 2.5 mg/kg, intramuscularly.

3.2.4. Cell Culture

3.2.4.1. Isolation of T. annulata *culture from peripheral blood mononuclear cells*

To detect the presence of macroschizonts in experimental calves, peripheral blood mononuclear cells (PBM) were isolated from 10 ml aliquots of peripheral blood using Ficoll-Paque (Pharmacia) as described previously by (Brown, 1987). Ten ml of blood in lithium-heparin was mixed with 10 ml of sterile phosphate buffered saline (PBS) pH 7.3 (BDH). The blood-PBS mixture was layered onto 8 ml of Ficoll-Paque, and centrifuged at 1,000 x g for 30 minutes at 15°C. The PBM were collected from the interface and washed in 20 ml PBS by centrifugation at 300 x g for 10 minutes at 15°C. The supernatant, containing the platelets, was decanted and the pellet was washed once more in 20 ml PBS and centrifuged as above but for 5 minutes. The cell pellet was resuspended in 10 ml cold complete medium, RPMI-1640 (Gibco) supplemented with 20% heat inactivated foetal calf serum (FCS), 2 mM L-Glutamine, 100 μg/ml streptomycin, 100 iu/ml penicillin, 5 x 10^{-5} M 2-mercapto-ethanol (2-ME). The suspension was put into a 25 cm^2 tissue culture flask and placed in a humidified incubator (LEEC) gassed with 5% CO_2 at 37°C with the cap loose. Giemsa's stained cytospin slides were prepared to check the quality of the isolated cells and for the presence of macroschizont infected cells.

Following isolation of the PBM from blood, the medium was changed every second or third day by centrifuging 8 ml cell suspension at 300 x g for 5 minutes at room temperature and removing the medium before resuspending the cell pellet in 8 ml of fresh warmed medium as described above and then returning it to the original flask. Each time a cytospin smear was prepared to assess whether infection had established. When more than 50% of cells were infected, the cells were passaged into a new flask. Once parasitosis reached 80% or more 2-ME was omitted from the

medium. PBM cell cultures were kept for at least three weeks before discarding. Figure 3.1 shows an established macroschizont infected cell line.

3.2.4.2. Maintenance of cell cultures

Established cell lines were maintained by reducing the number of cells, which increase by Log_{10} every 48-72 hours, by appropriate dilution in fresh medium (Brown, 1987). Cell growth was assessed using an inverted microscope and cytospins, subculturing was carried out by diluting the cells 1:10 in order to start cultures fresh at a cell density of 1-2 x 10^5 per ml in culture medium containing 10% FCS. The PBM cell culture flasks were kept for at least three weeks before deciding a cell line had not been established.

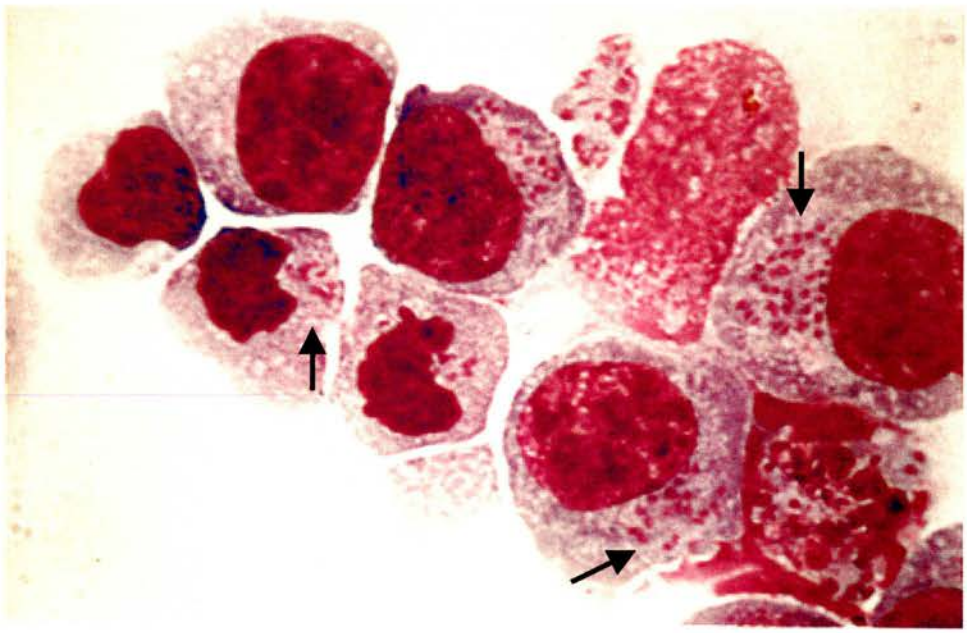
3.2.4.3. Cryopreservation of cell lines

Cells from two day old cultures in logarithmic growth phase at a density of 1-2 x 10^6 cells/ml were cryopreserved in 10% dimethyl sulphoxide (DMSO, Analar BDH) in culture medium containing 20% FCS (Brown, 1987). The culture was centrifuged at 300 x g for 5 minutes, and then resuspended in cold complete medium without 2-ME at 2 x 10^7 cells/ml. An equal volume of ice-cold 20% DMSO in complete medium, was added dropwise, keeping the culture on ice. One ml aliquots of cell suspension were aliquoted into cooled 1 ml cryovials (Nunc, Gibco). The cryovials were kept at –80°C for 24 hours and then transferred into a liquid nitrogen refrigerator (BOC) for long term storage.

3.2.4.4. Resuscitation of cell lines

Resuscitation of *T. annulata* infected cell lines was carried out by the method described by Brown (1987). Cells were thawed rapidly in a 37°C water bath and immediately pipetted into 8 ml of warm complete RPMI-1640 with 20% FCS. Cells were then centrifuged at 300 x g for 5 minutes at room temperature, to dilute out the DMSO. The cells were resuspended in 5 ml fresh complete medium and incubated at 37°C. The following day a further 5 ml medium was added and the cultures maintained as described above.

Figure 3.1. Macroschizont infected cells in a cytocentrifuge smear of *T. annulata* infected cell culture (x 1000, Giemsa stain). Arrows show the macroschizonts in the mononuclear cells.



3.2.5. Polymerase Chain Reaction

3.2.5.1. DNA preparation from blood

Blood samples were collected into EDTA vacutainer tubes and one ml was kept at –20°C until use. Blood samples were thawed at room temperature and centrifuged at 13,000 x g for 5 minutes and the pellet recovered. Pellets were resuspended in 1 ml saponin lysis buffer (0.22% NaCl, 0.015 % Saponin, (Sigma)) mixed on a vortex mixer and centrifuged as before. This was repeated twice and the last pellet washed in PBS. The pellets were resuspended in 100 µl of 1 x PCR reaction buffer (20 mM Tris-HCl pH 8.55, 16 mM $(NH_4)_2$ SO_4, 2.5 mM $MgCl_2$, 150 µg/ml BSA) containing proteinase K at a concentration of 100 µg/ml, mixed gently then incubated in a water bath at 56°C for one hour. Following heat inactivation at 95°C for 10 minutes the DNA samples were stored at –20°C until use.

3.2.5.2. PCR

Two primers, namely 989 and 1347, derived from the small subunit ribosomal RNA (ssu rRNA) gene of *T. annulata* were used to detect the parasite in the blood by PCR as described previously (Ilhan, 1995). The size of the expected amplification product was 372 bp. Primer 989 (5' AGT TTC TGA CCT ATC AG 3') is well conserved in the genus *Theileria* and primer 1347 (5' TGC ACA GAC CCC AGA GG 3') is specific to *T. annulata* (Allsopp *et al.*, 1993). PCR was performed in a final reaction volume of 100 µl containing 20 mM Tris-HCl pH 8.55, 16 mM $(NH_4)_2SO_4$, 4.0 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dNTP) (Promega), 2.5 U of *Taq* polymerase (Thermometric), 0.4 µM primers and 5 µl of DNA template. The PCR was performed using an automatic DNA thermal cycler (Hybaid) operating on a regimen of 95°C for 4 minutes, then 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute for 40 cycles followed by 72°C for 10 minutes.

3.2.5.3. Detection of PCR products

a) Agarose Gel Electrophoresis

Amplified products were detected on a Midi system (Bio-Rad) using 1.6% agarose gel (Ultra pure DNA grade agarose, Sigma) in Tris-Borate EDTA buffer (TBE) as described by Sambrook *et al.* (1989). Sixteen µl of each sample was mixed

with 4 µl of 6x DNA loading buffer and loaded onto the gel. A 1 Kb DNA ladder (Gibco) was used as a base-pair size marker. Electrophoresis was performed in 0.5 x TBE at 80V for 1.5 hours. Gels were stained in 0.5 µg/ml ethidium bromide (Amresco) for 30 minutes, de-stained in 80 mΩ water produced with a Milli-Q system (Millipore) for 30 minutes, amplification products were viewed under ultra-violet (UV) illumination and photographed using Polaroid black-and-white film, type 55 (Sigma).

b) Southern Blot Hybridisation

Southern blotting

The sensitivity of the amplification of the 372 bp *T. annulata* DNA fragment was confirmed by Southern blot hybridisation. DNA from the agarose gel was transferred to a nylon membrane (Hybond N+, Amersham) using the capillary transfer method as described by Sambrook *et al.* (1989). Briefly, DNA was denatured following electrophoresis by soaking the gel in three changes of alkali denaturing solution (pH 8.0) over 45 minutes. The gel was rinsed briefly with 80 mΩ water and then neutralised in three changes of neutralisation solution. Transfer of DNA from the gel to the nylon membrane was achieved by capillary movement of a high salt buffer (20 x SSC, pH 7.0) overnight. The DNA was cross-linked to the membrane by exposure to 150 Joules of short wave UV radiation using GS Gene Linker (Bio-Rad). The membrane was kept at 4°C until use.

Purification of probe from agarose gel

The "Gene Clean 2" kit (BIO 101) was used to recover the predicted 372 bp PCR product of purified piroplasm DNA from agarose gels. The method is based on the use of a powdered silica matrix to bind DNA, excess agarose is then washed away (Vogelstein and Gillespie, 1979). The desired bands were visualised under UV illumination, excised from the gel, and dissolved in three volumes of 3 M NaI at 50°C in the water bath. Five µl of silica matrix in water (glassmilk) was added, the mixture was vortexed then incubated at room temperature for 5 minutes. Following centrifugation at 9,000 x g for 5 seconds, the pellet was washed three times with "NEW wash" (a solution of NaCl, Tris, EDTA and ethanol) (BIO 101). DNA was then eluted in 5 µl sterile 80 mΩ water at 50°C for 3 minutes and DNA in the

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supernatant was removed after centrifugation at 9,000 x g for 30 seconds. Elution was carried out twice and the DNA obtained was kept at –20°C until use.

DIG DNA labelling and detection

The DNA fragments were labelled with digoxigenin-11-dUTP and visualised with an immunological detection system using the DIG DNA Labelling and Detection kit (Boehringer Mannheim) according to manufacturer's instructions. DNA was denatured by heating at 95°C for 10 minutes in boiling water. The labelling reaction consisted of 7 µl denatured DNA, 2 µl of hexanucleotide mix, 8 µl 80 mΩ water, 2µl of dNTP mixture and 1µl (2 units) of Klenow enzyme. The mixture was incubated at 37°C in a water bath overnight. Labelled DNA was then stored at –20°C for further use.

Hybridisation

Hybridisation was carried out according to manufacturer's instructions. Nylon blots were prehybridised in hybridisation buffer at 42°C for 1 hour. Hybridisation was carried out by incubating the blots at 42°C overnight in fresh buffer containing the appropriate DIG labelled DNA probe. Before addition, labelled probes were denatured by boiling for 10 minutes. Two µl of labelled probe was added to 2-5 ml hybridisation buffer per 100 cm^2 membrane. Standard DNA markers (1 Kb ladder) which were DIG labelled as described were also added to some blots.

Blots were washed in two changes of 2 x SSC, 0.1% SDS at room temperature (2 x 5 minutes), then 0.1 x SSC, 0.1% SDS at 68°C under constant agitation (2 x 15 minutes).

Immunological detection

Immunological detection was carried out according to manufacturer's instructions. Membranes were washed briefly in washing buffer (100mM maleic acid, 150 mM NaCl pH 7.5, 0.3% Tween 20) for 5 minutes, then incubated for 30 minutes in 1% blocking buffer (1% blocking reagent in 100mM maleic acid, 150 mM NaCl pH 7.5). The membrane was incubated for 30 minutes in anti-digoxigenin-alkaline phosphatase conjugate at a dilution of 1:5,000 (150 mU/ml) in blocking buffer. The antibody solution was decanted and the membrane was washed in washing buffer (2 x 15 minutes), then equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl pH 9.5) for 2-5 minutes. The membrane was incubated in

chromogen-substrate solution (0.34 mg/ml NBT, 0.18 mg/ml BCIP in detection buffer) until strong bands appeared without background staining. The reaction was stopped by adding distilled water. The result was documented by photography using Polaroid black-and-white film, type 55.

3.3. RESULTS

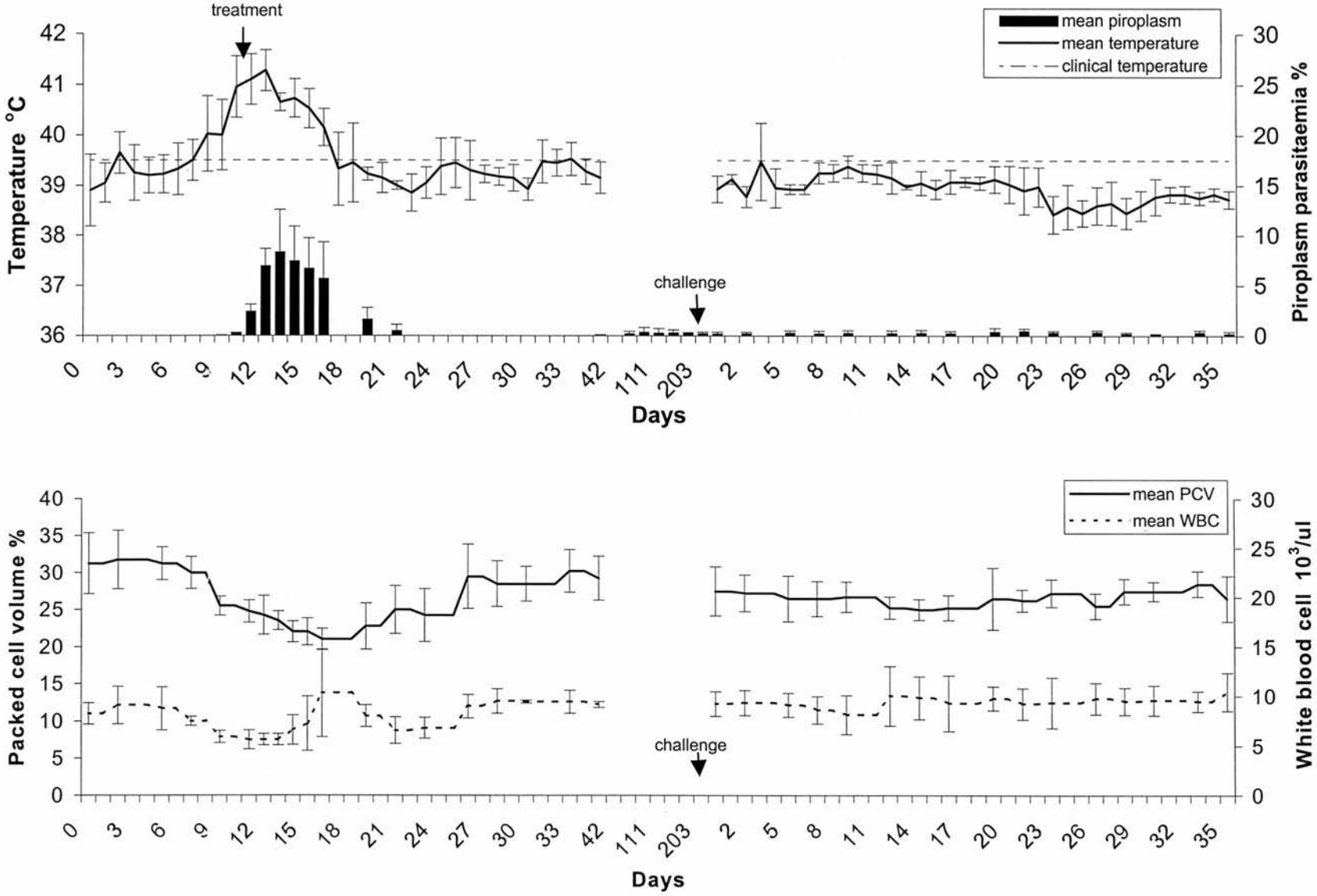
3.3.3. Clinical Reaction of Calves to Infection and Challenge

The haematological, parasitic and febrile reactions of calves are given individually in Figures 3.2 to 3.7. The severity of reaction was determined using a combination of clinical, parasitological and haematological parameters adapted from the recommendation made for *T. parva* (Anon, 1989). Parameters assessed included condition of the animal, duration of pyrexia occurring in the presence of macroschizonts, percentage and duration of macroschizont parasitosis and piroplasm parasitaemia, % reduction in PCV and white blood cell count. When the temperature remained above 39.5°C for 6 days or more, macroschizont and piroplasms were above 5% and 10% respectively and PCV was below 20%, the reaction was classified as severe. Any animal requiring buparvaquone (Butalex) treatment was considered to have been undergoing a severe reaction.

When macroschizont parasitosis and piroplasm parasitaemia were below 1% and 5% respectively, the duration of febrile period was less than 3 days and no significant reduction in PCV occurred, the reaction was classified as mild. Between these parameters for severe and mild reactions the reaction was called moderate.

Group 1 (infection with *T. annulata* Ankara sporozoites): All calves showed a similar severe clinical reaction to infection with 7 to 12 days of pyrexia, a slight decrease in WBC numbers. All calves showed reduction in PCV values (Figure 3.2). Macroschizont parasitosis was observed for 9 to 13 days (data not shown). Calves were treated either on day 12, 13 or 14 after infection at a single dose of 2.5 mg/kg of buparvaquone (Figure 3.2).

Figure 3.2. Clinical, parasitological and haematological reaction of calves (n=4) infected with sporozoites (Group 1) to infection and challenge 7 months after primary immunisation. *T. annulata* Ankara sporozoite stabilate 54 was used to infect calves. One ml of the sporozoite suspension containing 0.2 tick equivalent was inoculated subcutaneously in front of the right shoulder of each calf. *T. annulata* Gharb sporozoite stabilate 63 and 67 were pooled and used to challenge the calves at a dose of 1 tick-equivalent seven months after primary infection. During primary infection all calves which underwent severe reaction were treated with buparvaquone at a dose of 2.5 mg/kg. Mean values are given together with the standard deviation of the mean.


treatment
challenge
mean piroplasm
mean temperature
clinical temperature
Temperature °C
Piroplasm parasitaemia %
Days
mean PCV
mean WBC
Packed cell volume %
White blood cell 10^3/ul
Days

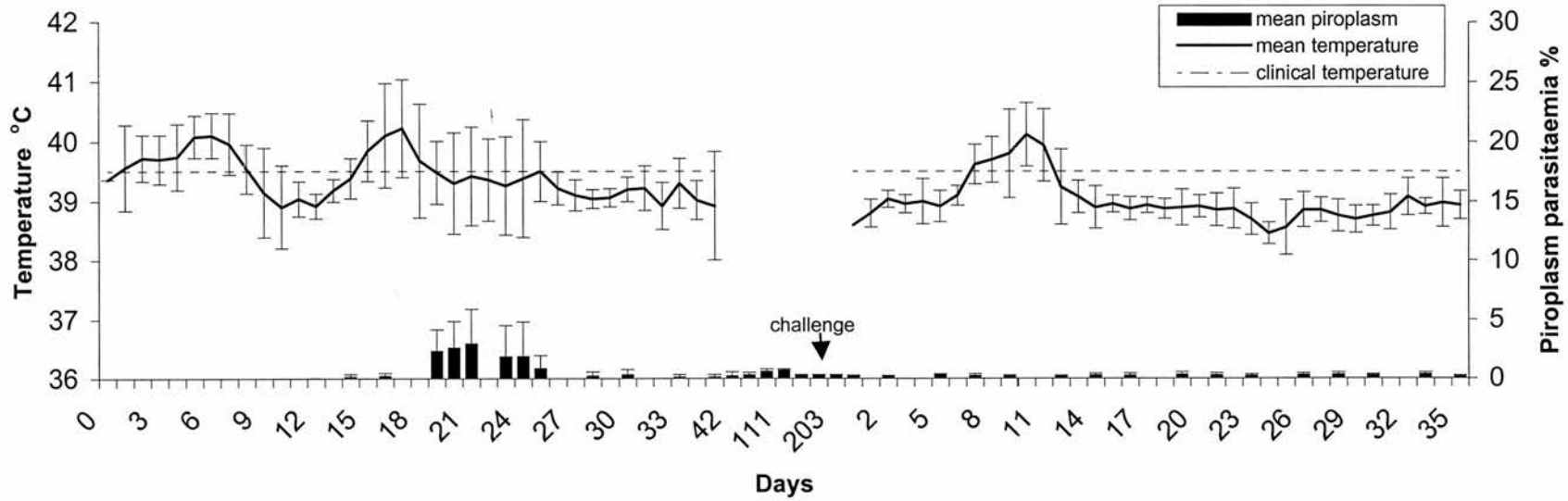
Following the challenge, all calves showed a mild reaction with body temperature, PCV and WBC remaining around the pre-infection values except on day 9 when calf 21A showed slight pyrexia. Increase in piroplasm parasitaemia was not significant (Figure 3.2). Macroschizont parasitosis was detected for 1 to 6 days (data not shown).

Groups 2A and 2B (immunisation with low passage *T. annulata* Ankara cell line): Two peaks of temperature were observed. The first peak was thought to be the reaction to the cells inoculated. The second peak was accepted as the reaction to the parasite infection. Calves in these groups exhibited a wide range of reactions from severe to mild. Two calves (32C, 892A) showed a severe reaction with 4 to 10 days pyrexia and macroschizont parasitosis was observed 7 to 8 days. Both calves required treatment. One calf (calf 22A) showed a mild reaction with one day of pyrexia and no macroschizont in the lymph node smears. The remaining six calves exhibited moderate reactions with 3 to 7 days of pyrexia (Figures 3.3 and 3.4). All calves showed reduction in PCV and WBC. Macroschizont parasitosis was observed for 1 to 7 days.

Following challenge, calves in Group 2A (low passage cell line for 7 months) showed a mild reaction with 5 to 7 days of pyrexia, a slight decrease in WBC numbers, no change in PCV and piroplasm levels (Figure 3.3) while macroschizont parasitosis was detected for 7 or 8 days (data not shown). All calves in Group 2B (low passage cell line for 1 month), except calf 15 exhibited a mild parasitic reaction with a brief pyrexia and macroschizonts being detected for one day. Calf 15 showed pyrexia for 12 days. There were no other obvious symptoms of *T. annulata* infection. The PCV, WBC and piroplasm parasitaemia remained close to pre-immunisation levels (Figure 3.4).

Groups 3A and 3B (high passage *T. annulata* Ankara/Pendik attenuated or 'vaccine' cell line): No calves in these groups showed any evidence of infection except one or two day(s) of pyrexia which was recorded in calves 32A, 308A and 54C (Figure 3.5 and 3.6). Macroschizonts and piroplasms were not detected. PCV and WBC remained around the pre-infection values.

Figure 3.3. Clinical, parasitological and haematological reaction of calves (n=5) immunised with the low passage cell line (passage 5) (Group 2A) and challenged 7 months after primary infection. A single dose of 2 x 10^6 live cells, which obtained from two day-old mononuclear cell culture infected with *T. annulata* Ankara (p5) schizonts, were inoculated subcutaneously in front of the right shoulder. *T. annulata* Gharb sporozoite stabilate 63 and 67 were pooled and used to challenge the calves at a dose of 1 tick-equivalent seven months after immunisation. Mean values are given together with the standard deviation of the mean.


mean piroplasm
mean temperature
clinical temperature
Temperature °C
Piroplasm parasitaemia %
challenge
Days

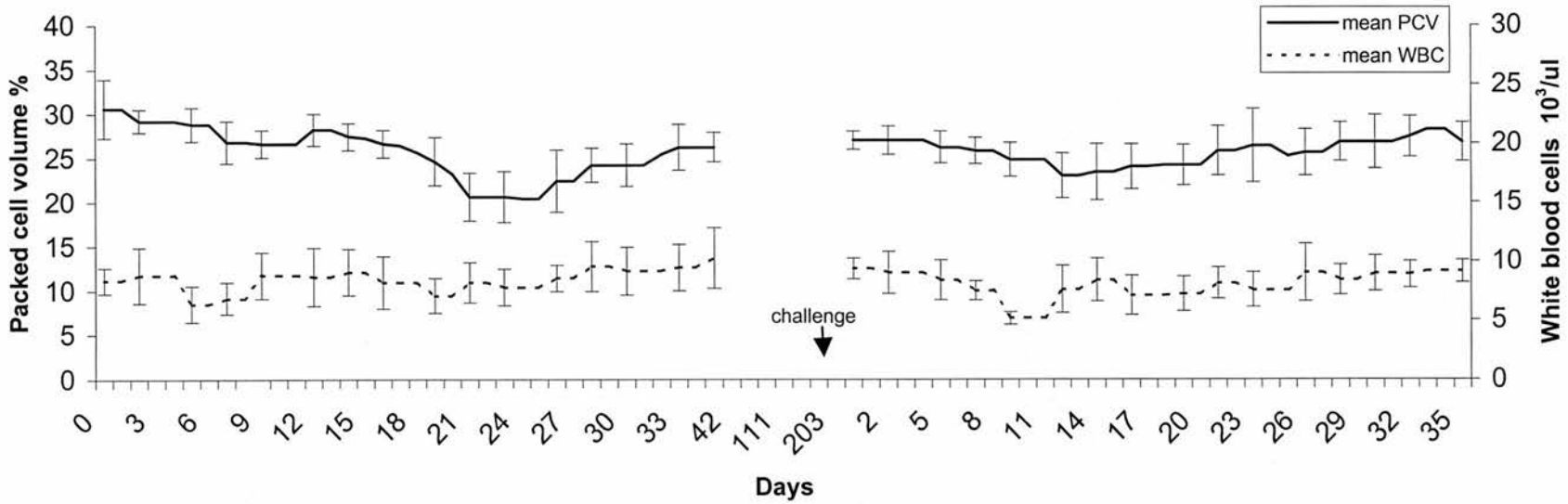
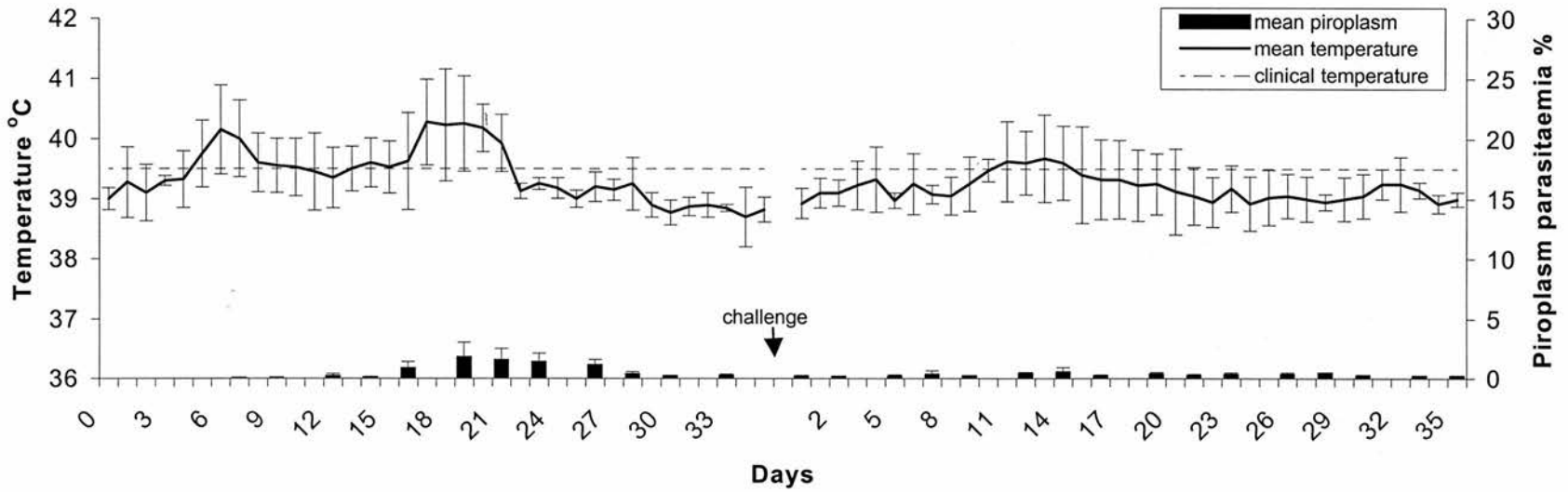

mean PCV
mean WBC
Packed cell volume %
White blood cells 10³/ul
challenge
Days

Figure 3.4. Clinical, parasitological and haematological reaction of calves (n=4) immunised with the low passage cell line (passage 5) (Group 2B) and challenged one month after immunisation. A single dose of 2 x 10^6 live cells, which obtained from two day-old mononuclear cell culture infected with *T. annulata* Ankara (p5) schizonts, were inoculated subcutaneously in front of the right shoulder. *T. annulata* Gharb sporozoite stabilate 63 and 67 were pooled and used to challenge the calves at a dose of 1 tick-equivalent 35 days after immunisation. Mean values are given together with the standard deviation of the mean.


mean piroplasm
mean temperature
clinical temperature
Temperature °C
Piroplasm parasitaemia %
challenge
Days

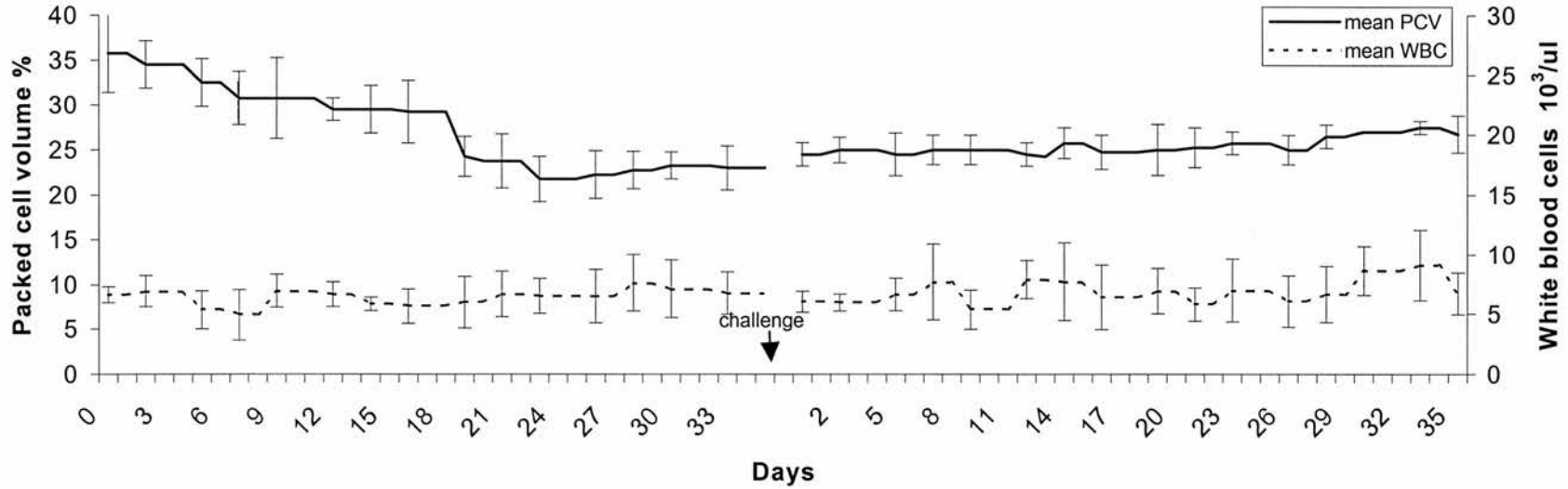
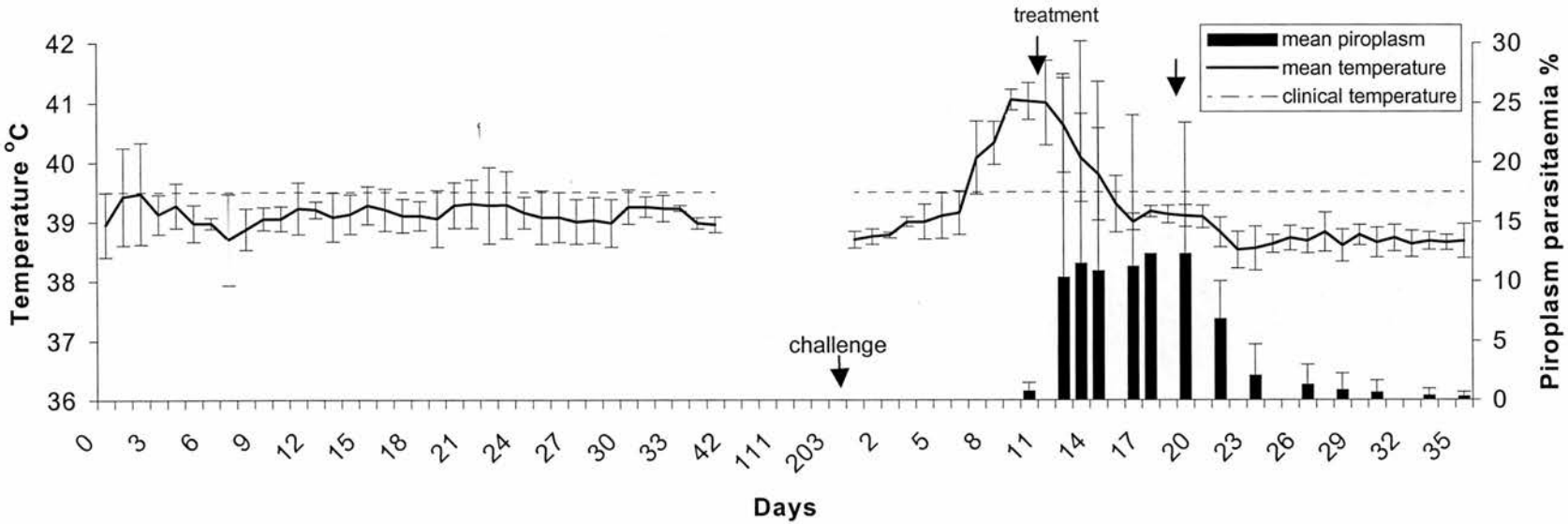

mean PCV
mean WBC
Packed cell volume %
White blood cells 10³/ul
challenge
Days

Figure 3.5. Clinical, parasitological and haematological reaction of calves (n=4) immunised with the high passage cell line (passage 317) (Group 3A) and challenged 7 months after immunisation. A single dose of 2 x 10^6 live cells, which obtained from two day-old mononuclear cell culture infected with *T. annulata* Ankara/Pendik (p317) schizonts, were inoculated subcutaneously in front of the right shoulder. *T. annulata* Gharb sporozoite stabilate 63 and 67 were pooled and used to challenge the calves at a dose of 1 tick-equivalent seven months after immunisation. During challenge infection calves (54C and 891A) which underwent severe reaction were treated with buparvaquone at a dose of 2.5 mg/kg. Mean values are given together with the standard deviation of the mean.


treatment
mean piroplasm
mean temperature
clinical temperature
Temperature °C
Piroplasm parasitaemia %
challenge
Days

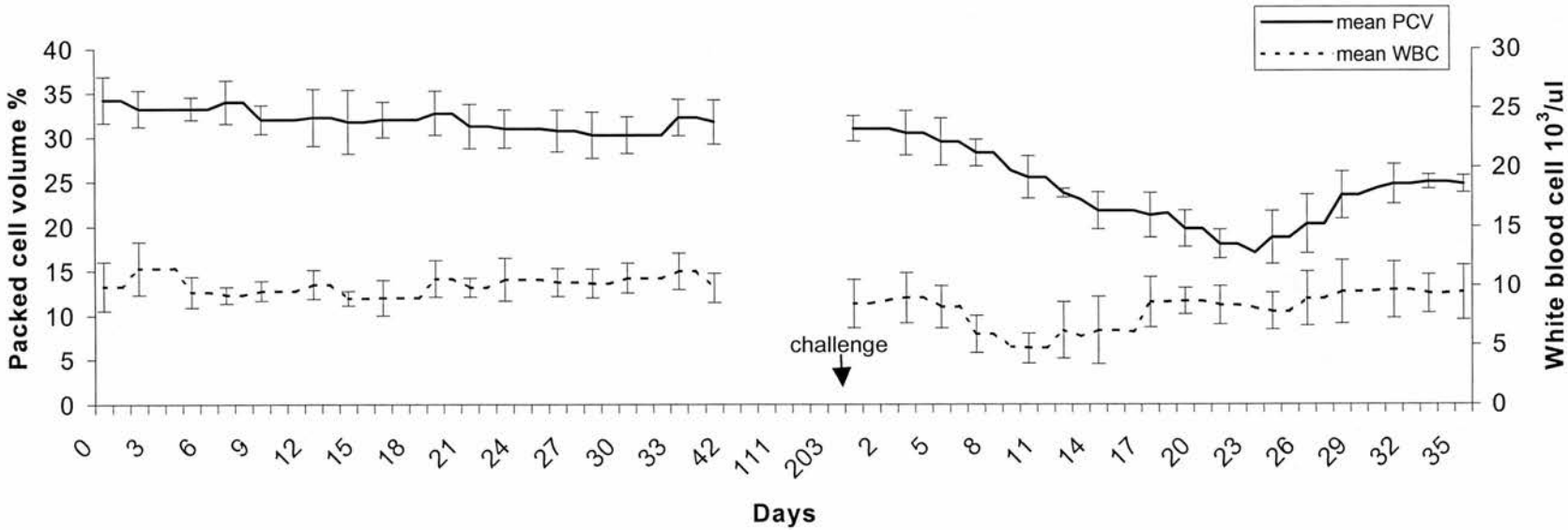
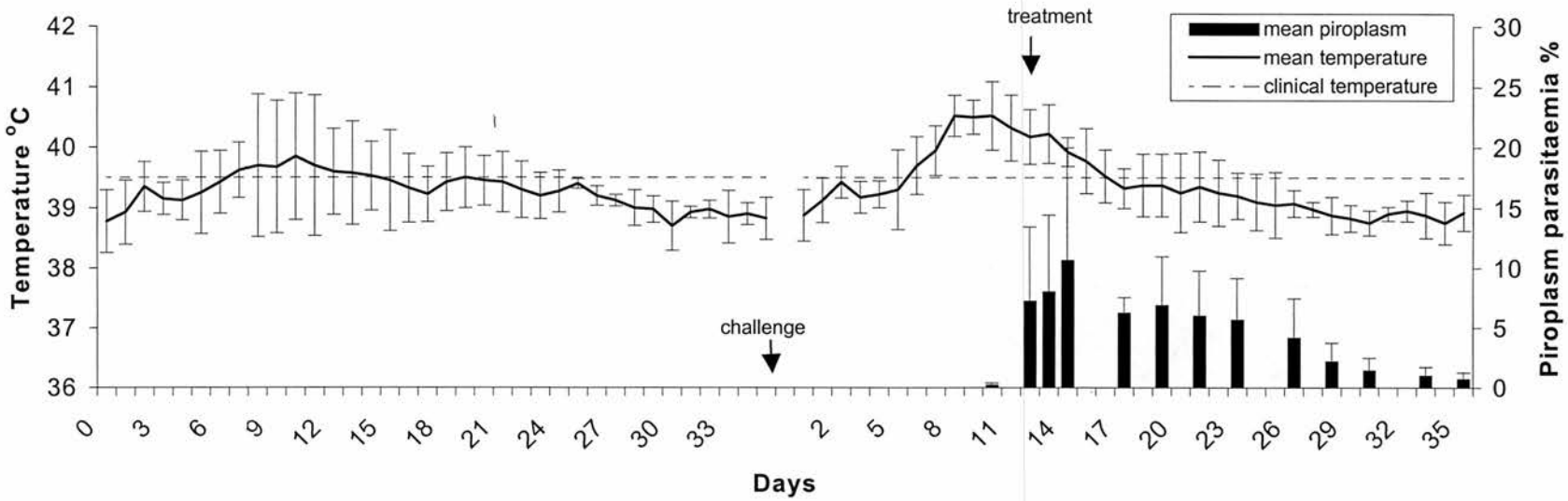
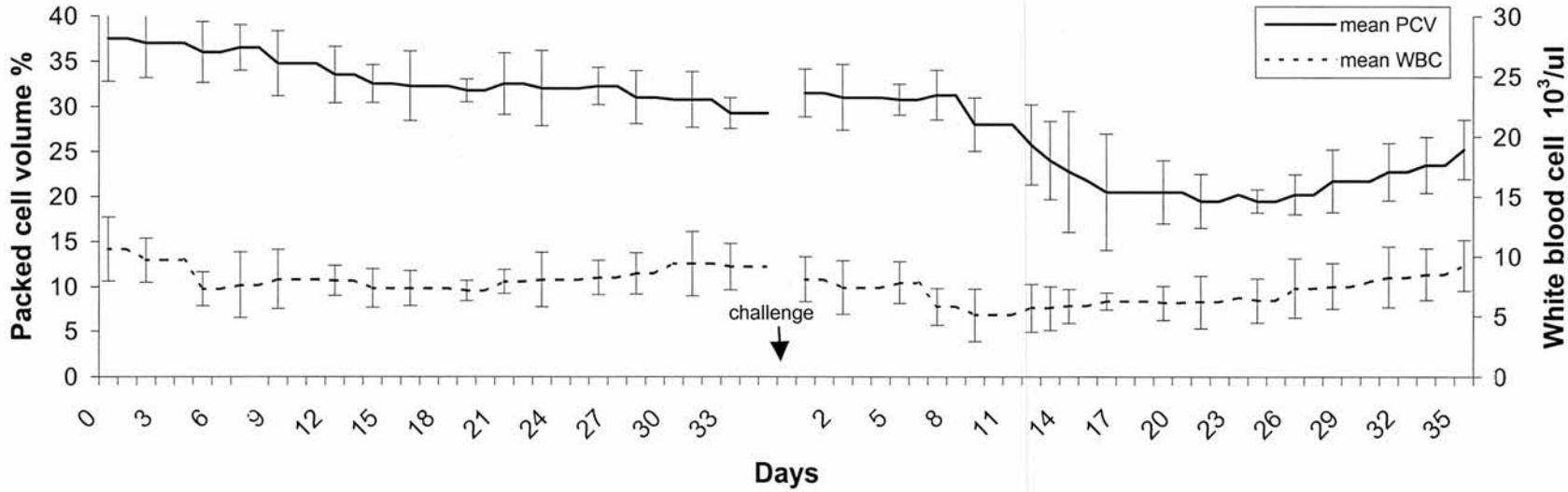

mean PCV
mean WBC
Packed cell volume %
White blood cell 10³/ul
challenge
Days

Figure 3.6. Clinical, parasitological and haematological reaction of calves (n=4) immunised with the high passage cell line (passage 317) (Group 3B) and challenged one month after immunisation. A single dose of 2 x 10^6 live cells, which obtained from two day-old mononuclear cell culture infected with *T. annulata* Ankara/Pendik (p317) schizonts, were inoculated subcutaneously in front of the right shoulder. *T. annulata* Gharb sporozoite stabilate 63 and 67 were pooled and used to challenge the calves at a dose of 1 tick-equivalent one month after immunisation. During challenge calf 308A which underwent severe reaction was treated with buparvaquone at a dose of 2.5 mg/kg. Mean values are given together with the standard deviation of the mean.


treatment
mean piroplasm
mean temperature
clinical temperature
challenge
Temperature °C
Piroplasm parasitaemia %
Days


mean PCV
mean WBC
challenge
Packed cell volume %
White blood cell 10³/ul
Days

Following challenge, calves in Group 3A (attenuated cell line for 7 months) showed moderate to severe clinical and parasitological reactions with pyrexia lasting for 6 to 10 days (Figure 3.5). Macroschizont parasitosis was observed for 8 to 10 days. Maximum piroplasm parasitaemia was 39.4% in calf 54C, 11.8% in calf 891A, 7.2% in calf 26A and 2.7% 32A. PCV dropped to less than 20% in all calves. Reduction in WBC numbers was observed in all calves. Calves 54C and 891A required treatment on day 12 and 21 after challenge, respectively.

The responses to heterologous challenge of calves in Group 3B (attenuated cell line for 1 month) were different. Calf 308A showed a severe reaction and required treatment on day 14 after challenge at a dose of 5 mg/kg buparvaquone (Figure 3.6). Pyrexia remained for 10 days, piroplasm parasitaemia reached to 26.4%, PCV fell to 12% and the WBC count was reduced. Whereas the remaining three calves in this group underwent a moderate reaction with pyrexia lasting for 9 to 19 days, piroplasm parasitaemia was around 10% and a decrease in the WBC and PCV. The calf recovered without treatment. Macroschizonts remained for 10 days in both calves with a high percentage of infected lympoblasts in calf 308A (Figure 3.6).

Group 4 (susceptible controls): After infection all calves underwent a severe clinical reaction. The decrease in PCV and WBC were notable and all calves developed severe anaemia. The maximum level of piroplasm parasitaemia was between 36.7 and 59.9%. Calves suffered a long period of pyrexia and macroschizont parasitosis. Calves were treated three times with buparvaquone starting from day 12 (Figure 3.7). Two calves showing respiratory system disorders were treated with an antibiotic (Synulox) applied intramuscularly for 5 days.

3.3.5. Parasitological Examination

The mean piroplasm parasitaemia of calves in each group is shown in Figures 3.2-3.7. The number of animals that were positive for piroplasms is shown in Figure 3.8. The data shown in Figure 3.8 are obtained on days during which PCR samples were taken. Calves in Group 1 became positive on day 9 and remained positive until and after challenge except following treatment between days 21 and 28 (Figure 3.2). Calves in 2A and 2B became piroplasm positive on day 7 or 16 and remained

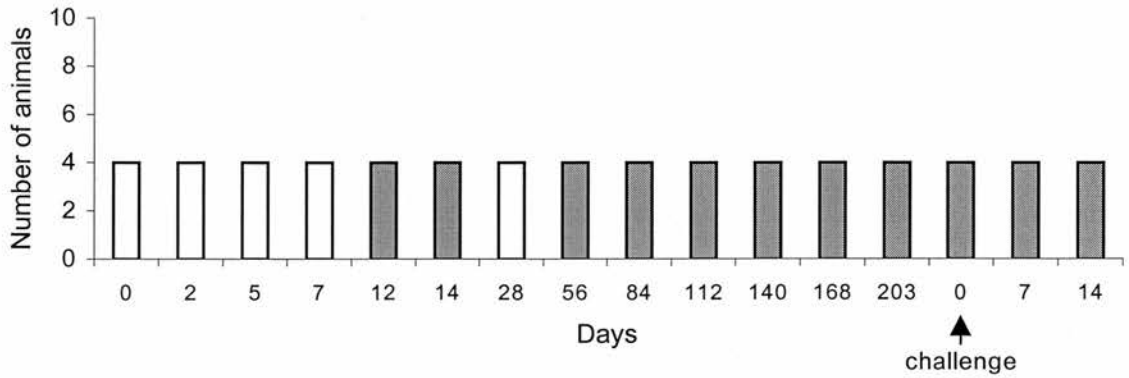
Figure 3.7. Clinical, parasitological and haematological reaction of calves (n=4) (Group 4) to challenge infection. *T. annulata* Gharb sporozoite stabilate 63 and 67 were pooled and used to infect the calves at a dose of 1 tick-equivalent. One ml of pooled stabilate was inoculated subcutaneously over the front of the left shoulder of each calf. Rectal temperatures were recorded daily. Animals with temperature above 39.5 were considered to be febrile. All calves, which underwent severe reaction, were treated with buparvaquone at a dose of 2.5 mg/kg. Mean values are given together with the standard deviation of the mean.

Piroplasm parasitaemia %
70
60
50
40
30
20
10
0
mean piroplasm
mean temperature
clinical temperature
treatment
Temperature °C
42
41
40
39
38
37
36
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Days

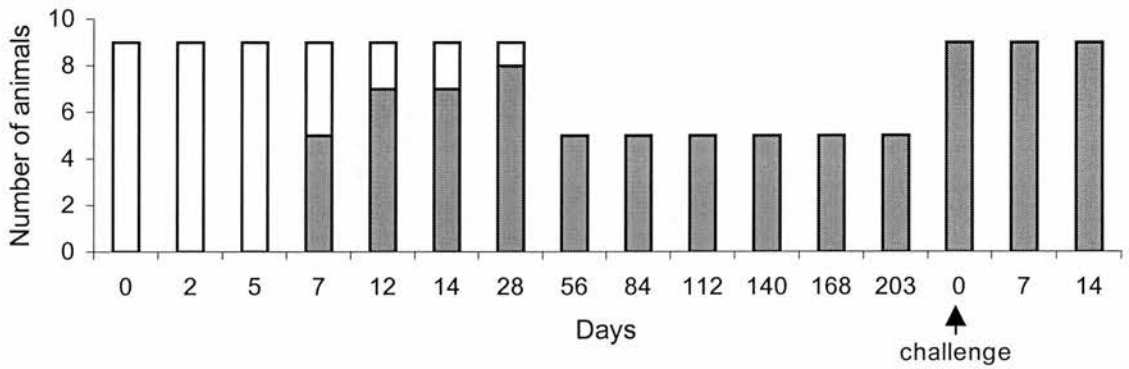
White blood cell 10³/ul
30
25
20
15
10
5
0
mean PCV
mean WBC
Packed cell volume %
40
35
30
25
20
15
10
5
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Days

Figure 3.8. Detection of carrier state in immunised and infected calves by microscopic examination of piroplasm parasitaemia. Solid bars indicate number of animals that were piroplasm positive. Open bars indicate number of animals that were piroplasm negative. **A.** Number of positive and negative animals that were infected with sporozoites and challenged seven months after primary infection. **B.** Number of positive and negative animals immunised with the low passage cell line (Groups 2A and 2B). Blood samples were taken from animals for seven months in Group 2A and for one month in Group 2B before challenge and 14 days after challenge in both groups. Data for Groups 2A and 2B are combined for the first 28 days of immunisation and throughout challenge. **C.** Number of positive and negative animals immunised with the high passage cell line (Groups 3A and 3B). Blood samples were taken from animals for seven months in Group 3A and for one month in Group 3B before challenge and 14 days after challenge in both groups. Data for Groups 3A and 3B are combined for the first 28 days of immunisation and throughout challenge.

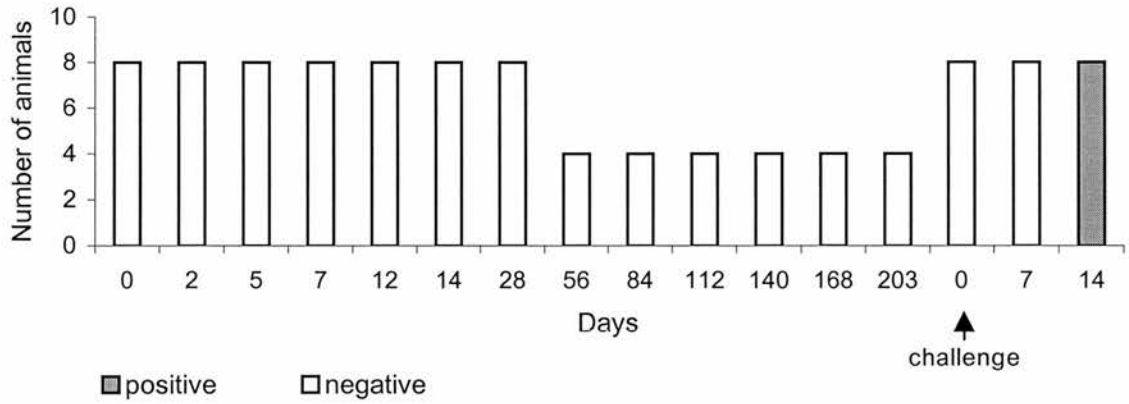
A. Sporozoite infection (Group



B. Low passage cell line immunisation (Groups 2A and 2B)



C. High passage cell line immunisation (Groups 3A and 3B)



positive until and after challenge (Figure 3.3-3.4). Following treatment no piroplasms were detected in calf 892A between day 28 and 35. In contrast, piroplasms were not detected in any calves in Groups 3A and 3B following primary infection until challenge. Following challenge all calves became piroplasm positive by day 10 (Figure 3.5-3.6).

3.3.6. Cell Culture Isolation

Results of attempts to establish infected cell cultures from PBM are given in Figure 3.9. Cell lines could be cultured for up to day 56 after inoculation from calves infected with sporozoites and for up to 28 days after inoculation from calves immunised with the low and high passage cell lines.

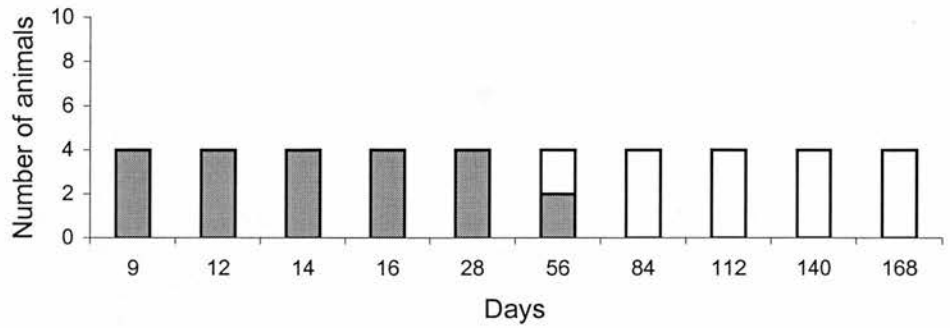
3.3.7. Detection of *T. annulata* Infection in Calves by PCR

T. annulata DNA was detected in blood obtained from experimentally infected and immunised calves by PCR, using primers 989 and 1347. PCR products were analysed by both electrophoresis on agarose gels and Southern blot hybridisation. Some blood samples gave positive results by hybridisation but were negative on the agarose gels (data not shown). No products were obtained in PCR reactions from negative control samples indicating that there was no contamination with extraneous *T. annulata* DNA.

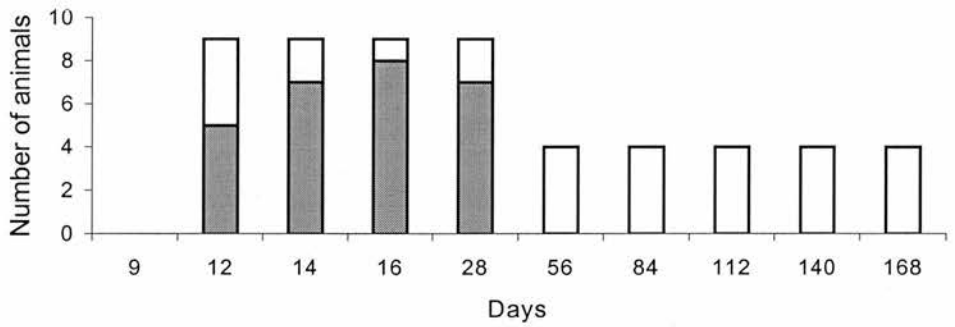
The number of positive animals in each group is given in Figure 3.10. Calves in Group 1 infected with sporozoites became positive on day 7 or 12 and remained positive up to and after challenge. Calves infected with the low passage cell line 7 out of 9 calves were positive on day 5, while the other calves in Groups 2 became positive on day 12 and remained positive up to and after challenge. Calves in Groups 3A and 3B showed intermittently positive PCR reactions. Generally the PCR products were weaker on agarose gel and were sometimes only positive on Southern blot hybridisation (data not shown). Only two calves were consistently positive by Southern blot hybridisation from days 12 to 35. Positive results did not always correlate with the results of blood smear examination or tissue culture. Following challenge all calves were positive on days 7 and 14.

Figure 3.9. Detection of carrier state in immunised and infected calves by isolation parasites in cell culture. Ten ml of jugular blood was taken into a vacutainer tube containing Lithium-Heparin. Isolation of *T. annulata*-macroschizont infected cells from peripheral blood mononuclear cells (PBM) was carried out as described in Materials and Methods. Solid bars indicate number of animals from which cell lines could be established. Open bars indicate number of animals from which cell lines could not be established. **A.** Number of positive and negative animals that were infected with sporozoites (Group 1) and tested for the establishment of cell lines for seven months. **B.** Number of positive and negative animals immunised with the low passage cell line (Groups 2A and 2B). Animals in Groups 2A and 2B were tested for the establishment of cell lines for seven months and one month, respectively. Data for Groups 2A and 2B are presented together for the first 28 days following immunisation. **C.** Number of positive and negative animals immunised with the high passage cell line (Groups 3A and 3B). Animals in Groups 3A and 3B were tested for the establishment of cell lines for seven months and one month, respectively. Data for Groups 3A and 3B are presented together for the first 28 days following immunisation.

A. Sporozoite infection (Group 1)



B. Low passage cell line immunisation (Groups 2A and 2B)



C. High passage cell line immunisation (Groups 3A and 3B)

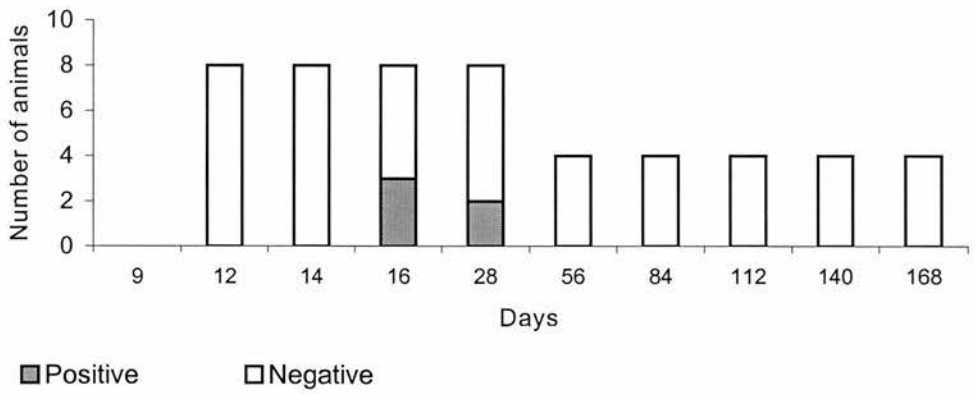
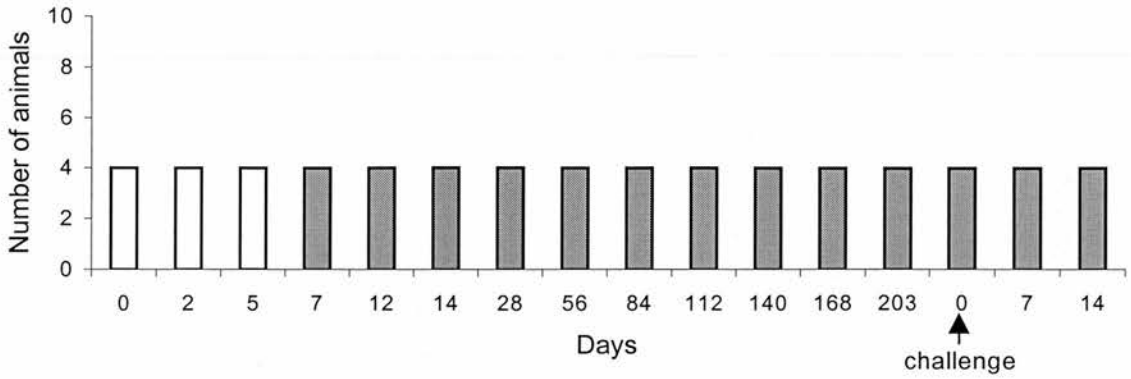
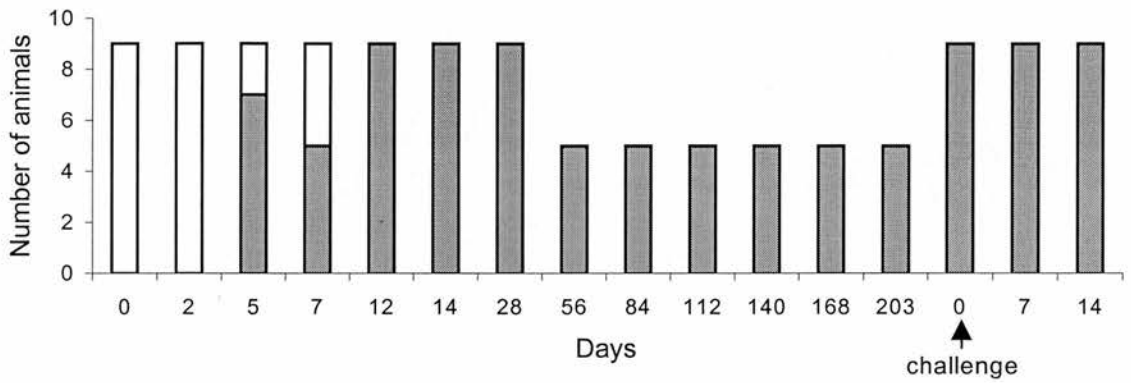


Figure 3.10. Detection of carrier state in immunised and infected cattle by polymerase chain reaction (PCR). DNA from blood samples was prepared using saponin lysis buffer as described in Materials and Methods. PCR primers derived from the small subunit ribosomal RNA gene of *T. annulata* were used to amplify a 372 bp fragment of *T. annulata*. PCR products were electrophoresed through a 1.6 % agarose gel and the presence of positive signals were confirmed by Southern blotting using the 372 bp amplification product. Solid bars indicate number of animals that showed positive signal. Open bars indicate number of animals that showed negative signal. **A.** Number of positive and negative animals that were infected with sporozoites and challenged seven months after primary infection. **B.** Number of positive and negative animals immunised with the low passage cell line (Groups 2A and 2B). Blood samples were taken from animals for seven months in Group 2A and for one month in Group 2B before challenge and 14 days after challenge in both groups. Data for Groups 2A and 2B are combined for the first 28 days of immunisation and throughout challenge. **C.** Number of positive and negative animals immunised with the high passage cell line (Groups 3A and 3B). Blood samples were taken from animals for seven months in Group 3A and for one month in Group 3B before challenge and 14 days after challenge in both groups. Data for Groups 3A and 3B are combined for the first 28 days of immunisation and throughout challenge.

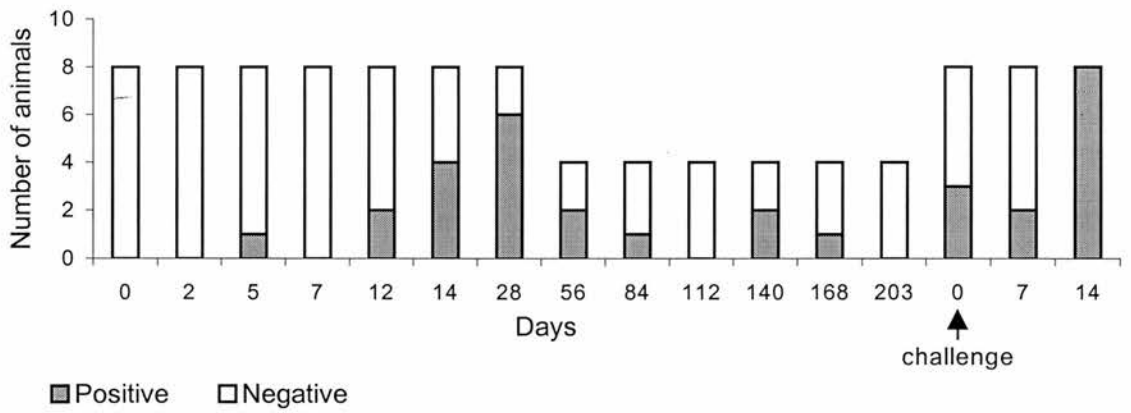
A. Sporozoite infection (Group 1)



B. Low passage cell line immunisation (Groups 2A and 2B)



C. High passage cell line immunisation (Groups 3A and 3B)



3.4. DISCUSSION

The data presented here compare differences in the immune response and the carrier state of animals infected with *T. annulata* sporozoites, low and high passage macroschizont infected cell lines. Differences in the severity of reaction to infection between groups indicated that sporozoite infection was most virulent, followed by the low passage cell line. The *T. annulata* Ankara/Pendik high passage cell line showed evidence of being highly attenuated, causing only very mild reactions. In Group 1, calves were challenged only seven months after primary sporozoite infection, but not one month after primary infection. All calves infected with sporozoites, in the experiment, required treatment with buparvaquone between days 12 and 14. It was previously shown that buparvaquone remains in the serum of calves at least 14 days following drug administration and reduces the severity of reaction produced by sporozoite infection (Wilkie *et al.*, 1998a). In addition, it was shown that serum samples taken from animals 14 days after drug administration reduced the infection rate of the cells by sporozoites *in vitro* (Wilkie *et al.*, 1998b). Therefore, challenging of calves one month after primary infection would not have demonstrated the real immune status of animals. The carrier state of parasite is also differed between the groups. Calves infected with sporozoites or immunised with the low passage cell line became piroplasm carriers following infection or immunisation. No piroplasms and macroschizonts were detected in Giemsa's stained blood and lymph node biopsy smears, respectively, in calves immunised with the high passage cell line.

The virulent heterologous sporozoite challenge indicated that different immunising parasite stages and levels of attenuation resulted in different levels and duration of immunity. The protective immunity that developed after inoculation of sporozoites and the low passage cell line was solid throughout the study. In contrast, the immunity induced by the high passage cell line was weaker. These results are in agreement with two lines of evidence suggesting that the degree of protection is controlled by the parasite stages and the level of attenuation. First, infection with sporozoites may stimulate better protection than that obtained by immunisation with cell lines (Preston and Brown, 1988). It is suggested that this is because cattle infected with sporozoites respond to sporozoite challenge immediately, whereas

those immunised with cell lines respond only after the development of schizonts (Preston *et al.*, 1999). Second, while macroschizonts provide optimal immunity to homologous challenge, they provide a lesser degree of protection against heterologous challenge (Sergent *et al.*, 1945; Barnett, 1963; Gill *et al.*, 1981; Subramanian *et al.*, 1986; Darghouth *et al.*, 1996a).

The different levels of immunity induced by inoculating sporozoites, low and high passage cell lines may be due to the exposure of animals to different stages of the parasite. The host's immune system responds to various stages of the parasite in different ways (Preston *et al.*, 1999). There is evidence indicating that antibodies against sporozoites and merozoites delays or prevents the invasion of mononuclear cells (Gray and Brown, 1981; Preston and Brown, 1985) or erythrocytes (d'Oliveira *et al.*, 1997a). Nevertheless, a subunit vaccination with the sporozoite surface antigen, SPAG-1, did not provide satisfactory protection alone, though it delayed the onset of disease (Boulter *et al.*, 1995; 1998). Similar results were obtained in vaccination trials using two allelic forms of the major merozoite surface protein, Tams1-1 and Tams1-2, (d'Oliveira *et al.*, 1997a).

The antigenic diversity between the parasites used to immunise and challenge calves might also play a role in the differences observed between immunisation regimes. Calves infected with sporozoites and the low passage cell line were exposed to several parasite populations as confirmed by glucose phosphate isomerase (GPI) polymorphism and by anti-macroschizont monoclonal antibodies (mAbs). In contrast, GPI analysis demonstrated that animals immunised with the high passage cell line had been infected with a single parasite population (Ilhan, 1995). Therefore, the exposure of animals to several parasite populations, rather than one, could provide better protection against a heterologous challenge. In the current study, the challenge with *T. annulata* Gharb was heterologous to all calf groups infected with *T. annulata* Ankara and resulted in effective protection of all calves infected with sporozoites or with the non-attenuated cell line. These results are in accordance with previous observations (Darghouth *et al.*, 1996a). In most cases animals in the field are exposed to a heterologous challenge by ticks. Therefore, vaccine cell lines that contain only one parasite population might not provide adequate protection against theileriosis.

The low level of immunity obtained with the high passage cell line could be due to the loss of immunogenicity and infectivity of the parasite through long term culture *in vitro*. The aim of attenuation is to reduce the virulence of the parasite while maintaining its immunogenicity and infectivity (Brown *et al.*, 1978a). Long term culture not only reduces the pathogenicity of cell lines, but also impairs their ability to protect against heterologous challenge (Pipano, 1989b). Prolonged maintenance in cell culture is also thought to result in altered gene expression, which might be responsible for the loss of virulence (Sutherland *et al.*, 1993). At present, however, it is difficult to identify a single factor that explains the effect of attenuation by long-term cell culture on immunogenicity.

The level of immunity obtained through the use attenuated cell lines depends on several factors including the ability of the parasite to transfer, establish and multiply within host cells (Brown *et al.*, 1978a). Attenuation reduces the ability of cell lines to produce detectable levels of macroschizont-infected cells in infected calves (Pipano, 1977). In the current study, cell culture could be established from 6 out of 8 calves in the high passage cell line infected animals during the first 28 days of immunisation. Additionally, the infected cells were shown to derive from the recipient animals by BoLA typing (Ilhan, 1995, data not shown), indicating that macroschizonts from the attenuated cell line transferred to the recipient host cells. Thus it is unlikely that the lack of protection in animals vaccinated with the high passage cell line is due to failure of the parasite to transfer into host cells.

It has been demonstrated that attenuated *T. annulata* Ode cells disseminate very poorly compared to virulent parent cell lines in *scid* mice (Somerville *et al.*, 1998a). The degree of dissemination was correlated with a marked reduction in matrix metalloproteinases (MMP), which are known to play an important role in metastasis (Sier *et al.*, 1996). Attenuation led to the loss of MMP activity in all vaccine lines examined, including the Ankara/Pendik cell line (Somerville *et al.*, 1998b). It has been suggested that the loss of MMP could be related to the loss of virulence of attenuated cell lines (Baylis *et al.*, 1992; Adamson and Hall, 1997; Somerville *et al.*, 1998b). These results suggest that the decreased immunogenicity of attenuated cell lines may be due to relatively low invasive ability of macroschizonts leading to reduce priming of the immune system.

There is very little information on the establishment and maintenance of macroschizonts in cattle after vaccination with attenuated macroschizont-infected cells. Traditionally, the immunisation status of vaccinated animals has been monitored using IFAT which does not reveal or necessarily imply the carrier state of animals or the presence of macroschizont infected cells (Pipano, 1974; Pipano, 1977). In the current study, the carrier status of animals was investigated by microscopic examination, cell culture isolation and PCR. During the current experimental study, animals infected with sporozoites or the low passage cell line were shown to be persistent carriers following infection by PCR and the presence of piroplasms in blood smears. In addition, cell cultures were established more frequently from these animals than animals immunised with the high passage cell line. In the latter group cell cultures could not be established 28 days after immunisation. Animals infected with the high passage cell line were rarely positive by PCR, microscopic examination or cell culture and did not present a persistent carrier state of piroplasms. However, it remains to be determined if the persistent parasite form following vaccination is a tissue-dwelling macroschizont within the reticulo-endothelial system. The classical definition of a carrier of *T. annulata* is the ability of an infected and recovered animal to serve as a reservoir of infection for vector ticks which are able to transmit the parasite to susceptible host (Young *et al.*, 1986). Currently the carrier status is thought to be maintained by the persistent and slow division of macroschizonts giving rise to piroplasms (Norval *et al.,* 1992). In addition, early studies described by Pipano (1972) indicate that infection could be established in splenectomised calves by inoculating red blood cells infected with piroplasms from animals 167 to 432 days after recovery. In the same study, macroschizonts were later detected in the recipient calves, implying the long-term persistence of macroschizonts developing to piroplasms.

It is interesting to note that in the current study animals that possessed solid immunity to challenge were also consistent piroplasm carriers throughout the experimental period up to challenge, while those that showed only sporadic or no evidence of being carriers were poorly protected from challenge. These observations suggest that there is a causal relationship between the level of immunity obtained and the carrier status of animals and that persistence of the parasite in animals are

necessary for the maintenance of immunity. The continued presence of the parasite might be necessary to reinforce immunological memory and to ensure an adequate and appropriate cellular immune response (Bunce and Bell, 1997). These findings support the notion of 'premunition' for *Theileria* and other protozoa as defined previously (Sergent, 1963).

Challenge with sporozoites under laboratory conditions may result in more severe reactions than challenge under natural conditions in the field (Pipano, 1995). For example, animals immunised with the vaccine cell line used in the current study, *viz.* Ankara/Pendik, at passage level 250, showed various reactions to laboratory challenge ranging from mild to severe/lethal (Ozkoc and Pipano, 1981). However, in a field study, the same vaccine cell line at passage level 252 to 325, was adequate for protection against field challenge (Onar, 1989). It is apparent that for most vaccine cell lines a slight loss in immunogenicity may not be very significant, unless animals are challenged with an unusually high dose or remain unchallenged for a long time following vaccination.

Previous studies indicated that there is no direct relationship between the severity of clinical reaction following immunisation with macroschizont infected cell lines and the level of protection (Innes, 1988). Brown *et al.* (1998b) demonstrated that the degree of clinical reactions following immunisation with cell lines may be related to the T-cell stimulatory ability of infected cells and the levels of pro-inflammatory cytokines they produce. In contrast to these observations, in the current study, calves that developed moderate or severe reactions to infection and immunisation were better protected against challenge than animals which exhibited only a mild reaction. These observations would suggest that a high level of parasitosis/parasitaemia during primary infection might stimulate a higher level of immunity.

Attenuated macroschizont infected cell lines have been used in many countries to control tropical theileriosis (Brown, 1990). However, the duration of protection, a key parameter in the design of vaccination programmes (Woolhouse and Bundy, 1997), remains largely unknown. In the current study, immunity stimulated by the Ankara/Pendik cell line (passage 317) protected three out of four animals one month following vaccination and two out of four 7 months following

vaccination. For vaccines with low impact and especially for those with relatively low duration of protection, repeated vaccination may be required according to the level of endemic stability in the region. However, re-immunisation with the same cell line may not boost immunity because of a graft rejection type response (Nichani *et al.*, 1997a; Nichani *et al.*, 1997b).

In subsequent chapters, the development and evaluation of ELISAs to detect *T. annulata* stage-specific antibodies will be discussed. The establishment of such ELISAs is essential to distinguish vaccinated animals from naturally infected animals and to detect which vaccinated animals have been challenged i.e. true prevalence of disease. Chapter 7 describes a nested PCR, which distinguishes *T. annulata* from *T. buffeli* in cattle and *T. annulata* from *T. lestoquardi and B. equi* in vector ticks. Such sensitive and specific tests are essential to target the limited amount of vaccine produced each year (150,000 doses in Turkey) to the areas of highest impact.

CHAPTER FOUR

EXPRESSION AND PURIFICATION OF MACROSCHIZONT STAGE SPECIFIC ANTIGENS

4.1. INTRODUCTION

At present the control of tropical theileriosis, in many countries where the disease is endemic, is by administration of attenuated live parasites to vaccinate cattle (Brown, 1990). In order to assess the impact of the control programs, it is necessary to be able to assess whether an animal has been vaccinated, whether it has been challenged in the field and whether it is immune to infection by *T. annulata*. One way of detecting vaccinated animals and monitoring a parasite challenge is the measurement and characteristics of their antibody responses against the parasite. Monitoring antibody responses before and after vaccination would provide information about the effectiveness of attenuated cell line vaccines, which have been used in defined areas. To date, the most commonly used system to monitor the efficacy of vaccines is by serological testing using the piroplasm and macroschizont IFAT (Pipano, 1974). However, IFAT fails to distinguish vaccinated/unchallenged animals from those that are either vaccinated/challenged or those that are simply naturally infected by ticks because of the presence of common antigens in all stages of the parasite (Knight *et al.*, 1998).

It has been shown for other parasite systems that stage specific antigens could be used to detect different stages of the infection. For example, a major surface glycoprotein of *Leishmania* parasites, gp63, seems to distinguish an ongoing infection from a previous one (Okong'o-Odera *et al.*, 1993). Similarly, a stage specific enzyme of *Schistosoma mansoni,* cercarial elastase, has been used as a sero-diagnostic marker for cercarial exposures during post-treatment follow-up and epidemiological studies (Ramzy *et al.*, 1997). Therefore, stage specific antigens of *T. annulata* might provide reagents that would allow vaccinated animals to be distinguished from naturally infected ones.

During a natural infection with *T. annulata* sporozoites, the host immune system will be exposed to sporozoites, macroschizonts, merozoites and piroplasms.

In contrast, animals immunised with high passage cell lines, which are believed not to produce piroplasms (Pipano, 1977), will be exposed only to the macroschizont stage of the parasite. Serological tests employing proteins specific to the sporozoite or piroplasm stages of the parasite could only detect animals infected with sporozoites, and thereby distinguish these animals from cattle vaccinated with these attenuated high passage schizont infected cell lines. To complement these antigens and further develop the assay, a recombinant protein specific to the macroschizont stage of the parasite could be used to detect both animals infected with sporozoites and animals that were vaccinated with an attenuated, high-passage cell line. In combination, these antigens would provide a portfolio of tests that could be used to measure the vaccination status of the cattle (using a macroschizont antigen), whether it had been recently challenged (using a sporozoite antigen) and the outcome of challenge (using a piroplasm antigen). The use of stage specific antigens would, therefore, be of great use to epidemiological studies investigating the true prevalence of the disease in areas where vaccine has been used as well as the assessment of the efficacy of the vaccination programme.

Recently, stage specific recombinant antigens from the sporozoite and the merozoite/piroplasm stages of the parasite have been defined. These include the sporozoite surface antigen, SPAG-1 (Williamson *et al.*, 1989; Hall *et al.*, 1992), the merozoite rhoptry antigen, Tamr-1 (Shiels *et al.*, 1994), and the 32 kDa major merozoite and piroplasm surface antigen, Tams1 (Shiels *et al.*, 1995). Preliminary, indirect ELISA studies were carried out using recombinant SPAG-1 and Tamr-1. The ELISA using SPAG-1 was not sensitive enough to detect antibodies in calves that had been exposed to sporozoites once (Matita, 1994; Williamson *et al.*, 1994). This indicated that the recombinant SPAG-1 protein would not be reliable in diagnostic tests aimed at detecting animals as positive after a single infection. In contrast, studies using recombinant Tamr-1 in an ELISA demonstrated that this protein was suitable as a reagent for a diagnostic test (Matita, 1994), as the Tamr-1 antigen was detected by the serum of the majority of infected animals. It was also demonstrated that antibodies against the Tamr-1 antigen were detected in animals infected with sporozoites or non-attenuated macroschizont infected cells; whereas the antibody titre of animals immunised with attenuated high passage cell lines remained close to

the pre-immunisation levels, or only increased slightly (Ilhan, 1995). The results of these experiments supported the plan of using stage specific recombinant antigens to distinguish vaccinated animals from naturally infected animals.

On commencement of this study, there were no recombinant antigens available that were expressed by the macroschizont stage of *T. annulata.* Such an antigen was needed for the development of a diagnostic system aimed at testing the efficacy of vaccination using attenuated cell lines. In order to clone a gene encoding a macroschizont antigen, gene expression libraries were screened with antisera raised against vaccine cell lines, following verification that the antisera detected an immunodominant macroschizont antigen. In addition, a subtractive screening procedure was employed using immune bovine sera whose antibodies that react with piroplasm antigens were blocked by immunoabsorption to increase the probability of isolating a macroschizont antigen gene. An alternative strategy was to identify homologues of macroschizont genes already cloned in related species of *Theileria.* As it is known that *T. annulata* and *T. parva* are closely related, the gene encoding the QP protein of *T. parva* (Baylis *et al.*, 1993) was considered to be a good candidate for this strategy. The QP antigen is synonymous with polymorphic immunodominant molecule (PIM) described by Toye *et al.* (1991; 1995b; 1996). The QP/PIM protein is expressed in the sporozoite, macroschizont and piroplasm stages of the parasite (Baylis *et al.*, 1993; Toye *et al.*, 1995b). PIM is one of the most immunogenic antigens of *T. parva* inducing strong antibody responses. It was demonstrated that a PIM-based ELISA for *T. parva* provided a high degree of sensitivity and specificity (99% and 94-98%, respectively) in detecting antibodies in cattle infected with *T. parva* (Katende *et al.*, 1998). Since PIM is expressed at all stages of the parasite, the *T. annulata* homologue of this antigen should detect antibodies in both naturally infected and vaccinated animals. Therefore, when used in combination with the Tamr-1 antigen, an ELISA using the *T. annulata* homologue of QP/PIM would allow one to distinguish vaccinated animals from those that were naturally infected or challenged following vaccination.

The primary aim of the work presented in this chapter was to isolate a gene(s) encoding an immunogenic protein that is expressed in the macroschizont stage of the parasite. Several approaches were considered to obtain such an antigen: i)

identification of the immunodominant molecules recognised by antibodies induced by vaccination using attenuated high passage cell lines; ii) screening of parasite genomic expression libraries with immune bovine sera whose antibodies against piroplasm antigens were blocked by immunoabsorption; iii) screening of cDNA and genomic DNA libraries with the gene encoding the QP protein of *T. parva.* These approaches were followed by expressing recombinant proteins encoded by the isolated genes in order to determine their immunogenicity.

4.2. MATERIALS AND METHODS

4.2.1. Parasite Material

The details of parasite material for the preparation of DNA, RNA and protein extracts analysed by Southern, northern or western blots are given in Table 4.1.

Cells from infected or uninfected cultures maintained as described in section 3.2.4.2 were centrifuged at 300 x g for 10 minutes at 4°C and the cell pellet was washed in PBS three times as above. Cells then were used to prepare DNA, RNA or protein.

The method described by Ben Miled (1993) was used to purify piroplasm from infected blood. Briefly, approximately 100 ml jugular blood from a *T. annulata* Ankara infected calf with a parasitaemia of 45% was taken into a sterile bottle containing 2000 IU sodium heparin. The blood was centrifuged at 2,500 x g for 15 minutes at 4°C. The plasma and buffy coat were removed and cells were washed three times in cold PBS while removing the buffy coat each time. The red cells were lysed at 37°C by addition of three volumes prewarmed 0.9 % ammonium chloride. After 10 minutes, when the colour of blood changed to deep red, the lysed erythrocytes were centrifuged at 800 x g for 5 minutes. The supernatant was centrifuged at 3,000 x g for 20 minutes at 4°C. The pellet containing free piroplasms was washed three times in cold PBS and used to prepare protein extract, DNA and RNA.

Table 4.1. Details of parasite material used in Southern, northern and western blot analyses.

Species	Stock	Stage	Country	origin/reference
T. annulata	Ankara	Sporozoite	Turkey	(Schein *et al.*, 1975)
T. annulata	Ankara	piroplasm	Turkey	(Schein *et al.*, 1975)
T. annulata	Ankara/ D7 clone	Schizont	Turkey	(Shiels *et al.*, 1992)
T. annulata	Gharb	Schizont	Morocco	(Ouhelli, 1985)
T. annulata	Hissar	Schizont	India	(Gill *et al.*, 1976)
T. annulata	Tova	Schizont	Israel	(Pipano *et al.*, 1974)
T. annulata	Beja	Schizont	Tunisia	(Ben Miled, 1993)
T. annulata	Razi	Schizont	Iran	(Hooshmand-Rad and Hashemi-Fesharki, 1968)
T. annulata	Soba	Schizont	Sudan	(Melrose *et al.*, 1984)
T. annulata	Caceres	Schizont	Spain	(de Kok *et al.*, 1993)
T. parva	Muguga	Schizont	Kenya	(Brocklesby *et al.*, 1961)
T. lestoquardi	Lahr	Schizont	Iran	(Hooshmand-Rad *et al.*, 1993)

4.2.2. SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Preparation of Protein Extracts

SDS-PAGE was carried out according to the method of Laemmli (1970), using the Biorad Protean II and mini Protean II gel electrophoresis systems. The acrylamide concentration of resolving gels varied from 8% to 10%, depending on the molecular mass range of the polypeptides analysed. For the mini Protean II apparatus, 5 ml of a 10% resolving gel was used routinely. The gel was composed of: 2 ml dH_2O, 1.7 ml acrylamide mix (30% acrylamide, 0.8 N-N Bis-methylene acrylamide, Sigma), 1.3 ml of 1.5 M Tris.HCl, pH 8.8 and 50 µl 10% SDS. Polymerisation was initiated by the addition of 50 µl freshly prepared 10% ammonium persulphate (APS, Sigma) and 2 µl tetra-methyl-1,2-diaminoethane (TEMED, Sigma). A 5% stack gel was poured on the top of the resolving gel, 2 ml for each gel, and an appropriate sample comb placed in the stacking gel. The 2 ml stack gel was composed of: 1.4 ml dH_2O, 0.33 ml 30% acrylamide mix, 0.25 ml 1M Tris.HCl pH 6.8 and 20 µl 10% SDS. When large Protean II gels were used the volumes of resolving and stacking gels were increased to approximately 30 ml and 5 ml respectively.

10 or 20 µl of protein extracts was loaded per lane. Molecular masses of proteins were estimated by reference to molecular markers of the high mass range (29-205 kDa, Sigma) or broad mass range (7-206 kDa, Biorad). Protean II gels were electrophoresed overnight in electrophoresis buffer (50 mM Tris base, 384 mM glycine, 2 mM EDTA and 0.1% SDS) at 50 V, while mini Protean II gels were run for approximately 2 hours at 120 V. Following electrophoresis, gels were stained and fixed in a solution of 30% methanol, 10% acetic acid and 0.5% Coomassie Brilliant Blue R-250 (Sigma) in dH_2O for approximately one hour and destained in the same solution lacking the Coomassie dye.

Piroplasms (100 µl pellet), macroschizont infected cells and BL-20 cells (5 x 10^7 cells) were lysed by adding 1 ml of 1 x sample buffer (diluted from 4x stock: 0.25M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.001% bromophenol blue). Protein samples were passed through a needle twice, boiled for 5 minutes and centrifuged at 14,000 x g for 5 minutes.

4.2.3. Western Blotting

Immunoblotting of polyacrylamide gels was adapted from (Towbin *et al.*, 1990). Following electrophoresis, proteins were transferred onto nitrocellulose filters (Schleicher and Schuel, BA 85, 0.45 μm or Hybond C, Amersham) in transfer buffer using either a Bio-Rad Trans blot cell wet system (25 mM Tris base, 192 mM glycine, 20% methanol) or semi-dry system (50 mM Tris base, 380 mM glycine, 0.1% SDS, 20% methanol). Transfer was carried out with a current of 300 mA for 1 or 3 hours for wet gels and for 30 or 60 minutes at 15-20V for the semi-dry system. The efficiency of transfer was determined by staining the filters for 5 minutes with 0.2% Ponceau-S (Sigma) in 3% trichloroacetic acid, followed by destaining in distilled water. Filters were washed with Tris-saline buffer (10 mM Tris.HCl, pH 7.4, 150mM NaCl, 0.1% Tween 20). Filters were then incubated in blocking buffer (Tris-saline buffer plus 5% nonfat dried milk (Marvel)) at room temperature on a rocking platform for one hour. Following blocking, filters were incubated overnight, or for 2 hours, at room temperature in primary antibody; bovine sera were diluted at 1:500 - 1:800 and rabbit sera were diluted 1:200 – 1:500 in block buffer. The filters were then washed three times for 5 minutes in Tris-saline buffer, to remove unbound antibodies, and incubated for one hour with anti-bovine (raised in rabbit) or anti-rabbit (raised in goat) alkaline phosphatase conjugated secondary antibody (IgG whole molecule, Sigma), diluted 1:20,000 in blocking buffer. The filters were washed as above and developed by incubation with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) (Kirkegaard and Perry Laboratories Inc.). The reaction was stopped by washing the filters in tap water.

4.2.4. Blocking the Detection of Piroplasm Antigens by a Rabbit Antiserum (C9m) Raised Against Merozoites

Western blots containing extracts from uninfected BL-20, the *T. annulata* Ankara D7 cell line and *T. annulata* piroplasms were pre-incubated with rabbit antiserum (C9m) raised against merozoites at different dilutions (1:10-1:500) and incubation times of 2-16 hours to block reactivity to non-schizont specific antigens before incubation with bovine sera N57, N58 and 155 (at a dilution of 1:800) infected with either low passage cell lines or sporozoites.

4.2.5. Immunoabsorption of Anti-piroplasm Antibodies from Immune Sera

Immunoabsorption was carried out to block anti-piroplasm antibodies from an immune serum by a method described previously (Dickson and Shiels, 1993). Proteins were extracted from a 0.5 ml piroplasm pellet using 2% SDS in 50mM Tris.HCl, pH 8.0. Following the shearing of the DNA, the extract was diluted 1:5 with NET buffer (0.5% Nonidet 40, 5mM EDTA, 150 mM NaCl, 10mM Sodium Azide, 50 mM Tris.HCl pH 8.0). 500 µl of the piroplasm extract was incubated overnight with immune bovine serum (1:800 dilution in 10 ml immunoblot blocking buffer). The following morning, a further 500 µl of the piroplasm extract was added to the serum sample which was incubated at room temperature for a further two hours. The serum and piroplasm extract mixture was used for western blot analyses and for screening a *T. annulata* genomic expression library.

4.2.6. Screening of *T. annulata* Genomic Expression Library

A genomic DNA expression library previously constructed in the λgt11 vector using DNA isolated from purified *T. annulata* Ankara (D7) merozoites was donated by Dr. J. Kinnaird (Department of Veterinary Parasitology, University of Glasgow). *Eschericia coli* strain Y1090 R^- (Stratagene) was used as host bacterium to plate out the λgt11 genomic expression library. Y1090 R^- was streaked out onto L-Broth (LB) plates (1% bactotryptone, 0.5% yeast extract, 1% sodium chloride, 10 mM magnesium sulfate, 1.5% bactoagar) containing 100 µg/ml ampicillin and 0.2% maltose and incubated overnight at 37°C. Overnight cultures were prepared the following day by picking single colonies into 5 ml of LB-media (as LB plates without bactoagar) containing ampicillin and maltose and incubated overnight on an orbital shaker at 37°C. A 50 ml culture was generated by inoculation with 0.5 ml of overnight culture and incubation in an orbital shaker at 37°C until an absorbance of 0.3-0.5 at 600 nm was reached. The cells were centrifuged at 800 x g for 10 minutes at 4°C, and the pellet was re-suspended in 5 ml of 10 mM $MgSO_4$.

The λgt11 genomic expression library was diluted to 7.5×10^4 pfu/ml (Plaque forming units) in SM buffer (50 mM Tris.HCl pH 7.5, 10 mM $MgSO_4$, 100 mM sodium chloride, 0.01% w/v gelatin). 1 ml of diluted library was mixed with 0.8 ml of Y1090 R^- in 10 mM $MgSO_4$ and incubated at room temperature for 15 minutes. 50

ml of top agarose [LB media containing 0.7% low EEO agarose (Sigma)] was then added and the mixture poured onto a large LB-plate (20 cm^2) containing 10 μg/ml ampicillin. Plates were incubated at 42°C for 3-4 hours until plaques just began to be visible. Nitrocellulose membranes, pre-soaked in 10 mM isopropythio-β-D galactosidase (IPTG, Gibco BRL), were laid onto the plates. The plates were transferred immediately to 37°C and incubated for a further 4 hours. Following incubation, filters were marked for orientation, lifted into immunoblot blocking buffer (5% Marvel, 10% Horse serum, 0.1 % Tween-20 in Tris-saline pH 7.4) and kept at 4°C overnight. The filters were then incubated with immune serum diluted at 1:800 in blocking buffer plus piroplasm lysates (see section 4.2.5) for 2 hours at room temperature, washed 3 times in Tris-saline buffer with 0.1% Tween-20 for 10 minutes each and incubated with alkaline phosphatase-conjugated anti-bovine IgG, diluted 1:20,000, for 2 hours. Washed filters were developed using BCIP/NBT.

4.2.7. cDNA Library Screening

A λ ZAP cDNA library, constructed by Dr. J. Kinnaird, representing RNA of a cloned *T. annulata* Ankara macroschizont infected cell line (D7), was used to screen for a gene homologous to the QP / PIM gene of *T. parva.* The XL1 Blue MRF' *Eschericia coli* strain (Stratagene) was the host bacterium used to plate out the library using overnight cultures as detailed by the supplier.

To screen the λ ZAP D7/37 library, 2.5 x 10^4 plaque forming units in SM buffer were absorbed onto 0.6 ml of XL1 Blue MRF' cells by incubation at room temperature for 15 minutes. 8 ml of top agarose [LB-media containing 0.7% low EEO agarose (Sigma)], that was kept at approximately 55°C, was added to the cells and poured onto 14 cm diameter LB- plates. Once set, plates were incubated at 37°C overnight and then chilled at 4°C for 1 hour. Plaque lifts were taken from three plates by laying nylon membranes (Colony/Plaque Screen Membranes, NEN-Life Science) on top of the plates for two minutes and the orientation of the filter was marked with a needle. Lifted membranes were placed (phage side up) onto Whatman 3MM filter paper that was previously soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 7 minutes followed by immersion for 2 x 3 minutes in neutralising solution (1.5 M NaCl, 0.5 M Tris.HCl, pH 7.4), and rinsing in 2 x SSC (150 mM NaCl, 15 mM

sodium citrate, pH 7.0), 0.1% SDS. The DNA was fixed to the filter by exposure to 150 Joules short wave ultra-violet radiation using a GS Gene Linker (Bio-Rad).

Following UV cross-linking the filters were prehybridised with 15 ml Church and Gilbert hybridisation buffer (Church and Gilbert, 1984) at 55°C for two hours using the Hybaid cylinder and hybridisation oven system (Hybaid). Approximately 25 ng of a denatured DNA probe was radiolabelled using a Random Priming DNA Labelling Kit (Boehringer-Mannheim Pharmaceuticals) according to the manufacturer's instructions. The labelled probe denatured by boiling for 3 minutes was added to the 15 ml of Church and Gilbert hybridisation solution. Hybridisation was performed overnight at 55°C. The nylon membranes were then washed three times with 1 x SSC, 5% SDS at 55°C for 15 minutes, before being expose to autoradiography film (Kodak X-O MAT) overnight in a X-ray film cassette at -70°C.

Positive plaques were identified, picked into 1 ml SM buffer containing 20 µl of chloroform and stored at 4°C. One negative plaque was also picked to serve as a negative control in subsequent rounds of screening. To re-screen putative positive plaques, 3,000 pfu were absorbed onto 500 µl of XL1 Blue MRF' bacteria, mixed with 2.5 ml top agarose and plated onto 90 mm diameter LB plates.

4.2.8. *In Vivo* Excision

Single plaques were picked from agar plates, transferred to sterile microfuge tubes containing 500 µl SM buffer and 20 µl chloroform and incubated overnight at 4°C. Overnight cultures of *E. coli* XL1 Blue MRF' and SOLR (Stratagene) cells were grown up in LB/maltose medium. 500 µl of each overnight culture was used to inoculate 50 ml LB-medium containing maltose (1:100 dilution) and incubated in an orbital shaker at 37°C. XL-1 Blue MRF' cells were grown until an OD_{600} of 0.5 was obtained, spun down at 1,000 x g and resuspended to give an OD_{600} of 1.0 in 10 mM $MgSO_4$. The SOLR cells were allowed to grow to an OD_{600} of 0.5-1.0, then were kept at room temperature until used.

200 µl of XL1-Blue MRF' cells were combined with 250 µl (approximately 1 x 10^6 pfu) of λZAP phage stock isolated as described in section 4.2.7 and 1 µl (approximately 1 x 10^6 pfu/µl) ExAssist helper phage (Stratagene). The mixture was incubated at 37°C for 15 minutes, after which 3 ml LB medium was added and the

samples were incubated in an orbital shaker for a further 2.5 hours at 37°C. The tubes were then heated to 70°C for 20 minutes and centrifuged for 15 minutes at 2,000 x g. The resulting supernatant containing rescued phage was decanted into sterile tubes and stored at 4°C for up to 2 months. 10 µl and 100 µl of rescued phage stocks were mixed with 200 µl of SOLR cells (OD_{600}= 1.0) and incubated at 37°C for 15 minutes. 300 µl of LB was added and the samples were incubated for a further 45 minutes at 37°C. 200 µl from each mixture was then plated out onto LB/ampicillin plates and incubated overnight at 37°C. Colonies (3-5) were selected the next day, grown up overnight and glycerol stocks and plasmid DNA prepared. The presence of DNA inserts was examined by restriction enzyme analysis as described in section 4.2.9.

4.2.9. Plasmid DNA Preparation, Restriction Enzyme Analysis and Purification of DNA Fragments

Bacteria from 1.5 ml of an overnight culture were pelleted by centrifugation at 15,800 x g for 30 seconds. The pellet was resuspended in 150 µl of 50 mM Tris.HCl pH 7.5, 10 mM EDTA, 100 µg/ml RNAse A, lysed by the addition of 150 µl 200 mM NaOH, 1% SDS and neutralised by the addition of 150 µl 1.32 M potassium acetate, pH 4.8. Samples were then centrifuged for 30 minutes at 15,800 x g and supernatants collected. DNA was precipitated from the supernatants by the addition of 2 sample volumes of ice-cold ethanol, and the precipitate pelleted by centrifugation for 30 minutes. After washing the pellets in 70% ethanol and air-drying, DNA samples were resuspended in 30 µl sterile distilled water and stored at –20°C.

0.2 to 1 µl of plasmid DNA was mixed with 2 µl of appropriate 10x React buffer (Gibco BRL) and 5-10 u of restriction enzyme (Gibco BRL), and the final volume of the reaction was brought to 20 µl with sterile dH_2O. The mixture was incubated at 37°C for 1 - 2 hours. In multiple digests, if all the restriction enzymes required the same reaction buffer, digests were carried out at the same time. Otherwise, each digest was sequentially performed using appropriate buffers. 5 x Ficoll-loading dye (100 mM EDTA pH 7.5, 22% Ficoll, 0.05% Bromophenol blue) was then added to a final concentration of 1x and samples were run on agarose electrophoresis gels according to standard protocols (Sambrook *et al.*, 1989). 0.7 -1%

agarose gels were prepared by melting agarose (Sigma) in an appropriate volume of either TBE or TAE. When cooled to hand temperature, ethidium bromide (Sigma) was added to a final concentration of 0.5 μg/ml. The agarose was poured into a casting frame and the gel was allowed to set at room temperature. Gels were electrophoresed at 50-100 V in either TBE or TAE buffer and the size of the DNA fragments estimated by comparison to a 1 kb marker ladder (Gibco BRL). Bands were visualised by exposure to short wave ultraviolet light (366nm). When DNA fragments were to be excised from gels for random prime labelling (section 4.2.7), low melting agarose (Sigma) was used and the gels run at a maximum of 60 Volts. Otherwise bands of interest were excised and purified either by using the QIAquick Gel Extraction Kit (Qiagen) or the "Gene Clean 2" kit (BIO 101), according to the manufacturer's instructions.

4.2.10. Ligation of DNA Fragments and Transformation of Competent Cells

To ligate purified DNA restriction fragments into restriction digested vector DNA, ratios of insert to vector of 3:1 and 1:1 were used. Insert and vector DNA was taken up to a total volume of 17 μl with dH_2O and 2 μl of 10 x T4 DNA ligation buffer (500 mM Tris.HCl, pH 7.8, 100 mM dithiothreitol, 1 mM ATP, 25 μg/ml bovine serum albumin) and 1-2 units of T4 DNA ligase (Gibco BRL) were added. The ligation mixture was incubated overnight at 16°C and used to transform competent *E. coli* cells. To prepare competent cells, 500 μl of overnight culture of *E. coli* XL1-Blue (Stratagene) was used to inoculate 50 ml LB medium and the culture incubated at 37°C, with shaking, until an absorbance of 0.2-0.5 at 600 nm was obtained. Cells were centrifuged at 1,000 x g for 10 minutes, resuspended in half the pellet volume using pre-chilled 50 mM calcium chloride and incubated for 20 minutes on ice. Cells were centrifuged as before, resuspended in a 10^{th} of the original volume of 50 mM calcium chloride and left on ice for several hours before use.

For transformations, 10 μl of the ligation reaction or 10 ng of plasmid DNA were mixed with 50 μl of competent cells, left on ice for 45 minutes and heat shocked for 2 minutes at 42°C followed by very brief cooling on ice. 200 μl of LB was added and the cells were incubated at 37°C for 30-45 minutes. Cells were then plated onto LB/ampicillin plates that were pre-streaked with 2.5 μl of 1 M IPTG and

50 µl 5-bromo-4 chloro 3 indolyl β-D galactoside, (X-gal, 10mg/ml in dimethylformamide, Gibco BRL). Plates were incubated overnight at 37°C, white recombinant colonies picked and overnight cultures prepared in LB/ampicillin medium the next day. The presence of inserts was checked by restriction enzyme digestion as described in section 4.2.9.

4.2.11. Automated DNA Sequencing

Samples for sequence analysis were generated by combining approximately 500 ng of DNA, 2 pmol of infrared fluorophore (laser dye IRD 800) labelled T7, M13 or SP6 primers, 2.5 µl of 10 x sequencing buffer (0.5 M Tris.HCl pH 9.3, 25 mM magnesium chloride), 1 µl of BioPro thermostable DNA polymerase (Bioline) and dH_2O to final volume of 17 µl. 2 µl of the dideoxy termination mix (180 µM 7-deaze-dGTP, 50 mM NaCl; 180 µM dCTP, 50 mM NaCl; 180 µM dATP, 50 mM NaCl; 180 µM dTTP, 50 mM NaCl) was placed into one of 4 corresponding thermocycler tubes together with 4 µl of the template/primer/enzyme mix. The top of each reaction mixture was covered with 30 µl mineral oil and the tubes were placed onto a thermocycler (Hybaid) for the following cycles: initially 95°C for 5 minutes; followed by 30 cycles of: 95°C for 30 seconds (denaturing step), 60°C for 30 seconds (annealing step) and 70°C for 1 minute (elongation step). After the cycling program was completed, 4 µl of stop buffer (95% formamide, 20 mM EDTA pH 7.6, 0.1% bromophenol blue, 0.1% xylene cyanol FF) was added to each reaction mix and stored at –20°C until use. Samples were denatured by heating to 95°C for 5 minutes prior to loading onto 6 % acrylamide gels. Gels were prepared by dissolving 21.0 g of urea in 6 ml of LongRanger™ gel mix, 6 ml of 10 x TBE (890 mM Tris.HCl, 890 mM boric acid, 20 mM EDTA) and 23 ml dH_2O, giving a total volume of 50 ml. The acrylamide solution was degassed before polymerisation was initiated by the addition of 25 µl TEMED and 250 µl freshly made 10% APS. The gel was then cast using 41 cm x 25 cm x 0.5 cm LI-COR gel plates and was left for approximately 60 minutes to set. Assembly of the LI-COR 4000 DNA sequencer apparatus was carried out according to the manufacturer's instructions (LI-COR). The gel was pre-run with 1 x TBE until a running temperature of 50°C was reached. 0.8 µl of each sample was

loaded and electrophoresis was carried out for approximately 12 hours. Sequence information was collected using computer software Base Imager™, LI-COR.

4.2.12. Exonuclease III Deletions

Promega's Erase-a-Base System was used to generate deletions allowing derivation of the complete sequence of the *Theileria* gene inserts isolated from the λ ZAP cDNA library. Plasmid DNA was prepared as described in section 4.2.9, except before the alcohol precipitation, a phenol/chloroform extraction step was carried out. 50 µl of closed circular DNA, in either pBluescript or pGEM 7zf+ vectors, was linearized with two different restriction enzymes that cut within the multiple cloning site, between the end of the insert to be deleted and the sequencing primer binding site. The first enzyme generated a 4-base 3' overhanging end, close to the sequencing primer binding site that was resistant to exonuclease III digestion. The second enzyme digestion generated a blunt end or 5' overhanging adjacent to the insert that was susceptible to Exo III. After the first digestion, a 2 µl sample was taken and run on a 0.8% agarose gel to check that the DNA was cut to completion. Following the second digestion, the plasmid DNA was precipitated with 2.5 volumes of ethanol and 1/10 volume of 3 M NaAc pH 5.3 and the pellet resuspended in 52 µl sterile dH_2O. 6µl 10x EXO III Buffer and 4µl dH_2O were added to 50 µl DNA and the sample heated to 36°C. 350 units of Exo III were mixed with the DNA and, at one minute intervals, 4 µl samples were removed into Eppendorf tubes on ice that contained 12.0 µl S1 nuclease mix (40mM potassium acetate, pH 4.6, 338mM NaCl, 1.35mM $ZnSO_4$, 6.8% glycerol, 60 units of S1 nuclease). The tubes were then incubated at room temperature for 30 minutes. 1.4 µl of S1 nuclease stop buffer was added to each tube, followed by incubation at 70°C for 10 minutes and a brief centrifugation. 5 µl samples from each time point were run onto a 1% agarose gel for size analysis. 2.6 µl Klenow mix (20mM Tris.HCl, pH 8.0, 100mM $MgCl_2$, 4 units of Klenow DNA polymerase) was then added to samples containing DNA fragments that exceeded the size of the vector alone, followed by incubation at 37°C for 10 minutes. Re-ligation of the deletions was carried out in a total volume of 20 µl containing 1 x T4 Ligase buffer and 1 unit of T4 Ligase at 16°C overnight. 10 µl of the ligation

products were then transformed into XL-1 Blue cells, and 5-10 colonies from each time point screened to select those containing deletions of appropriate sizes for sequence analysis.

4.2.13. Southern Blotting

4.2.13.1. Preparation of Genomic DNA

Genomic DNA was prepared from purified macroschizont infected cell cultures and piroplasms using a method described previously (Ben Miled *et al.*, 1994). 100 ml of macroschizont infected cell culture was grown to a density of 10^6 cells/ml and centrifuged at 1,500 x g for 10 minutes. The cell pellet, or 0.5 ml of a purified piroplasm pellet, was resuspended in 5 ml of 1 x SSC. 5 ml of 100 mM Tris.HCl pH 7.5, 100 mM NaCl, 10mM EDTA, 1% Sarkosyl was then added to the cell suspension and mixed carefully for 2-3 minutes to lyse the cells. Proteinase K was added to a final concentration of 100 µg/ml and the lysate incubated at 55°C for 2 hours. An equal volume of phenol saturated with TE was added and mixed for 30 min on a rotator. The sample was centrifuged at 2,000 x g for 10 minutes, the aqueous phase recovered and a further extraction carried out with an equal volume of phenol-chloroform (1:1). Finally, the DNA was extracted once with an equal volume of chloroform alone. The aqueous top phase was transferred to a fresh tube and this 1/10 volume of 3M sodium acetate pH 5.4 and 2.5 volumes of ethanol were added. DNA was precipitated at -70°C for 2 hours, pelleted by centrifugation for 10 min and washed with 70% ethanol. Excess ethanol was removed and the pellet was allowed to dry before resuspension in 600µl of TE.

4.2.13.2. Southern Blotting and Hybridisation

20 µg of cell line DNA or 10 µg of piroplasm DNA were digested with 20-40 U of an appropriate restriction enzyme. After adding 2 µl RNAse A, the final volume was made up to 40 µl with dH_2O and was incubated at 37°C overnight. 10 µl of 5x Ficoll-loading buffer was added, and the samples along with the 1 kb marker ladder were loaded onto a 0.8% agarose gel containing 0.5 µg/ml ethidium bromide and run at 35 Volts overnight. The gel was visualised under UV light.

Capillary transfer of DNA from agarose gel to nylon membrane was carried out according to standard protocols (Sambrook *et al.*, 1989). DNA in gels was denatured by immersion in 1.5M NaCl, 0.5M NaOH for 45 minutes on a shaker, then neutralised in 2 M NaCl 0.5 M Tris HCl, pH 7.4 for 30 minutes. The gel was then equilibrated in 25 mM phosphate transfer buffer, pH 5.5, for 30 minutes and the DNA was transferred onto nylon membrane (Gene Screen Hybridisation Transfer Membrane NEN^{TM}, Life Science Products, or Hybond N, Amersham) by capillary action for at least 18 hours. DNA was cross-linked to the membrane and hybridisation performed as described in section 4.2.7.

4.2.14. Northern Blotting

4.2.14.1. RNA extraction

Total RNA was prepared from macroschizont infected cell cultures or piroplasms using Tri-Reagent (Sigma). All solutions and equipment were treated with diethylpyrocarbonate (DEPC, Sigma) prior to use. 10^9 macroschizont infected cells or 500µl pellets of purified piroplasms were mixed with 4 ml of Tri-Reagent with a pipette until the lysate was fairly homogenous. After homogenisation, the suspension was centrifuged at 12,000 x g for 10 minutes at 4°C (Beckman, Model J2-21). The supernatant, containing RNA and protein, was transferred to a fresh tube and left to stand for 5 minutes at room temperature. 0.2 ml of chloroform was added per ml of Tri-reagent used. The samples were vortexed and allowed to stand for a further 10 minutes at room temperature. The samples were then centrifuged at 12,000 x g for 15 min at 4°C to separate the mixture into 3 phases. The colourless upper aqueous phase, containing RNA, was collected and 0.5 ml isopropanol added per ml of Tri-Reagent. After 10 minutes at room temperature, the RNA precipitate was pelleted by centrifugation at 12,000 x g for 10 min at 4°C and washed by adding 1 ml of 75% ethanol per ml of Tri-Reagent, followed by vortexing and centrifugation at 7,500 x g for 5 minutes at 4°C. The final pellets were dried for 5-10 minutes at 37°C, resuspended in 200 µl of 0.5% SDS and incubated at 55°C for 10 minutes. The concentration of each RNA sample was then measured by spectroscopy.

Total RNA was extracted from the salivary glands of approximately 200 *T. annulata* Ankara infected *Hyalomma anatolicum* nymphs, fed on rabbits for two

days (Williamson *et al.*, 1989; Hall *et al.*, 1992). The salivary glands were dissected under sterile conditions as described by Walker *et al.* (1979), put into 10 ml of Tri-Reagent (Sigma) and kept at –70°C. When the mixture was thawed, the tick salivary glands were homogenised using a Griffiths tube. After homogenisation, RNA was isolated as described for cell lines and piroplasms.

4.2.14.2. Northern blotting and hybridisation

RNA samples were loaded onto 1.2% agarose gel to assess the integrity of RNA. The gel tank (Pharmacia), combs and tray were thoroughly cleaned by washing with dH_2O and wiping with 70% ethanol. 195 ml dH_2O were mixed with 3.0 g of agarose low EEO (Sigma) and boiled in a microwave until the agarose was in solution. After cooling to hand temperature, 50 ml of 5x MOPS buffer (0.2M 3-[N-morpholino]-2-hydroxy-propanesulfonic acid (MOPS), pH 7.0, 50mM NaOAC, 5mM EDTA) and 5 ml of formaldehyde were added and the gel was allowed to set for 30 min. RNA samples (10 µg total RNA) were mixed with 4.5µl 5xMOPS buffer, 7.9µl 37% formaldehyde, 22.5µl formamide and RNAase free water (DEPC treated water) to a final volume of 45µl. Samples were denatured by heating to 55°C for 15 min and 5µl of dye mixture (95% formamide, 20 mM EDTA pH 7.6, 0.1% bromophenol blue, 0.1% xylene cyanol) were added after the samples were chilled on ice. The samples were loaded onto the gel together with a 0.24-9.5 kb RNA Ladder (Gibco BRL) and run at 100 Volts for 30 minutes followed by 35 Volts overnight in 1x MOPS buffer.

The gel was stained in 300 ml of dH_2O containing ethidium bromide (10µg/ml) for 30 minutes and then washed in two changes of dH_2O for 15 minutes each. Bands were visualised by exposure to short wave ultraviolet light (366nm).

Nucleic acids were transferred from gels to nylon membrane by capillary blotting using 25 mM phosphate transfer buffer. Gels were not stained with ethidium bromide in most cases except for the marker tract as ethidium bromide may interfere with transfer efficiency. Gels were washed with distilled water while the nylon membrane was soaked in transfer buffer (25mM phosphate buffer, pH 5.5) for 15 minutes. UV cross-linking and hybridisation were carried out as described in 4.2. 13.2.

4.2.15. Preparation of Expression Constructs

Both the pQE (QIAexpress, Qiagen Ltd,) and the pGex (Promega) vector systems were used to generate the expression constructs. In pQE vectors, the recombinant protein is tagged with six histidine residues (His_6) and in pGex it is tagged with the glutathione S-transferase (GST). Where appropriate restriction sites were available, subcloning was carried out directly into the correct reading frames of the vectors. Where there were no appropriate restriction sites in the DNA fragment to be cloned, oligonucleotide primers with suitable restriction enzyme sites were designed to flank the region to be expressed.

PCR was carried out at 94°C for 5 minutes (1 cycle); 94°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute (30 cycle) and 72°C for 10 minutes (1 cycle). PCR products were analysed on 1% agarose gels. The PCR products were ligated into the pGEM®-T Easy vector (Promega) using a 3:1 ratio of insert to vector DNA according to the manufacturer's instructions. Overnight cultures and plasmid DNA were prepared from 18 white colonies. In order to confirm the presence of the correct inserts, *Eco*RI digestions were carried out and examined by agarose gel electrophoresis. Positive inserts were confirmed by sequence analysis using T7 and SP6 universal primers. The inserts of the plasmid DNA were then released with restriction enzymes specific for the sites created by the primers and purified from agarose gels as described in section 4.2.9. Purified DNA was then ligated into either pQE vectors (-30, -31, -32) (QIAexpress) or pGex vectors (5x-1, 5x-2) (Promega).

4.2.15.1. Production of soluble purified recombinant antigen using pQE Expression Vectors

Ligations were transformed into M15 (pRep4) (Qiagen) competent cells which were plated out onto LB agar plates containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The presence of inserts was tested either by restriction digestion of DNA or by colony screening. Colonies were streaked out onto LB agar plates containing ampicillin and kanamycin and incubated for 8 hours at 37°C. Nylon membranes (Colony/Plaque Screen membranes, NEN-Life Science) were carefully placed onto the plates, left for two minutes and the orientation of the membranes marked with a needle. Once the membranes were lifted, plates were returned to the

37°C incubator and left overnight. Denaturation, neutralisation and fixation of the DNA on the filters were achieved as described in section 4.2.7. Hybridisation with a radiolabelled probe (section 4.2.7) was carried out at 65°C. Overnight cultures and plasmid DNA were prepared as described in sections 4.2.6 and 4.2.9. The cloning junctions and the open reading frame of each construct were confirmed by sequence analysis using vector specific primers. Screening of transformants for polypeptide expression was then carried out using small-scale cultures.

Single colonies of transformants were grown for 16 hours at 37°C in LB containing ampicillin and kanamycin.100 µl of each overnight culture was used to inoculate 3 ml LB with antibiotics, and the cells were allowed to grow at 37°C until they reached an OD_{600} of 0.7. A 500 µl aliquot was then removed as an 'uninduced' control sample and IPTG added (2mM) to the remaining culture to induce protein expression over a 5 hour period. 500 µl of cells were removed as "induced" sample. Control and induced cells were harvested by centrifugation at 15,000 x g for 20 seconds and resuspended in 200 µl of 1x SDS-PAGE sample buffer. All samples were boiled for 5 minutes and 20 µl aliquots analysed by 10% SDS-PAGE (see section 4.2.2.).

To analyse the solubility of the expressed protein, 2 ml of overnight culture were used to inoculate 100 ml of culture media and the cells were grown at 37°C, while shaking, until an absorbance of 0.7-0.9 at 600nm was reached. Induction was carried out as described above and cells were harvested by centrifugation at 4,000 x g for 10 minutes, followed by storage at –70°C. Pellets were thawed on ice, resuspended in 5 ml sonication buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl) and subjected to freeze thawing in dry-ice/ethanol and cold water to assist cell lysis. The suspension then was sonicated on ice for 5 cycles (20 second burst at maximum amplitude/20 second cooling periods) and centrifuged (10,000 x g, 20 minutes, 4°C). The supernatant was collected and the pellet was resuspended in 5 ml sonication buffer. Samples collected before induction, after induction and following sonication (supernatant and pellet) were analysed by 10% SDS-PAGE.

To purify recombinant protein under native conditions, a 500 ml culture OD_{600}=0.7 was generated and induced for 5 hours at 37°C by adding IPTG to 1 mM.

Cells were harvested by centrifugation (4,000 x g, 20 minutes, 4°C) and pellets stored at –70°C.

Thawed cells were resuspended in sonication buffer at 5 volumes per gram of wet weight and sonicated. DNAse I (0.5 μg/ml), phenylmethylsulfonyl fluoride (PMSF, 0.1 mM) and $MgCl_2$ (8 mM) were added and the lysate incubated for 10 minutes on ice. Following centrifugation at 10,000 x g for 20 minutes at 4°C, the supernatant was collected and mixed with 3 ml of a 50 % slurry of Ni-NTA resin (Qiagen), previously equilibrated in sonication buffer. The mixture was stirred at room temperature for 45 minutes and loaded into a 1.6 cm diameter column. The column was then washed with 100 ml sonication buffer and 100 ml wash buffer (50 mM Na-phospate pH 8.0, 300 mM NaCl, 10% glycerol, pH 6.0) and the protein eluted with 10 ml 0.1-0.5 M imidazole in Wash Buffer. 1 ml fractions were collected and analysed on 10% SDS-PAGE. The fractions containing the purified protein were then dialysed in several changes of PBS pH 7.4. The concentration of proteins was estimated using the BCA Protein Assay kit (Pierce) according to the manufacturer 's instructions. The protein concentration was calculated by spectrophotometric absorbance at 562 nm against a standard curve, established using bovine serum albumin standards ranging between 100 μg/ml and 2,000 μg/ml. The eluted proteins were divided into small aliquots (50 μl-1 ml) and kept at –70°C. For western blot analysis (see section 4.2.3), 1-10 μg protein was loaded per track.

If a recombinant protein was not soluble under the above extraction method the bacterial pellet was resuspended in the EGTA solution (0.25% Tween-20, 0.1 mM EGTA) and sonication carried out three times as described above. The supernatant was collected after each sonication and the pellet resuspended in fresh EGTA solution. SDS-PAGE and western blot analysis of supernatants and the final pellet was carried out using mouse anti-His-tag monoclonal antibody (Sigma). The primary anti- His -tag monoclonal antibody was used at a dilution of 1:3,000 and the secondary anti-mouse alkaline phosphatase conjugate (Sigma) at 1:10,000.

The methods described above were not always successful in generating soluble protein. Therefore, the method of Frangioni and Neel (1993) was also used. Following induction, cells from a 250 ml culture were pelleted and resuspended in 5 ml STE buffer (100 mM NaCl, 1 mM EDTA, 50 mM Tris.HCl pH 8.0) containing 1

mg/ml of lysozyme. PMSF was then added to a final concentration of 0.1 mM and the lysate was incubated at room temperature for 20 minutes with occasional stirring. $MgCl_2$ and DNAse I were added to a final concentration of 8 mM and 10μg/ml, respectively. The lysate was incubated for a further 30 minutes on ice and then sonicated. Triton X-100 was added to a final concentration of 2%, followed by centrifugation (15,000 x g for 20 minutes at 4°C). The supernatant was collected and fractionated on a Ni-NTA resin column as described above.

As a final method to obtain soluble recombinant antigen, purification of proteins under denaturing conditions was utilised. The bacteria were grown and induced as described above. Frozen bacterial pellets were thawed for 15 minutes at room temperature and resuspended in Buffer A (6 M GuHCl, 0.1 M Na-phosphate, 0.01 M Tris.HCl, pH 8.0) at 5 ml per gram wet weight. Cells were stirred for 11 hours at room temperature and the resulting lysates centrifuged at 10,000 g for 20 minutes at 4°C. The supernatant was then mixed with 3 ml of a 50 % slurry of Ni-NTA resin, previously equilibrated in Buffer A, stirred at room temperature for 45 minutes and loaded into a 1.6 cm diameter column. The column was washed with 10 column volumes of Buffer A, 5 column volumes of Buffer B (8 M Urea, 0.1 M Na-phosphate, 0.01 M Tris.HCl, pH 8.0), and 20 column volumes of Buffer C (8 M Urea, 0.1 M Na-Phosphate, 0.01 M Tris.HCl, pH 6.3). Protein was then eluted with 20 ml of Buffer F (6 M GuHCl, 0.2 M Acetic Acid). 3 ml fractions were collected and analysed by SDS-PAGE. Fractions containing the purified protein were then placed into dialysing tubing and the protein refolded by sequential dialysis against buffer with gradual dilution of Urea (6 M- 1M Urea, 0.05 M Tris.HCl, 0.005% Tween-80, 2 mM reduced glutathione, 0.02 mM oxidized glutathione, pH 8.0) (Jaenicke and Rudolph, 1990). The protein was dialysed overnight for each step. After the concentration of urea was reduced to 1 M, it was completely removed from the buffer. This was followed by the removal of glutathione and Tween-80 from the solution. The protein was finally dialysed against with two changes of 0.05 M Tris.HCl, pH 8.0. Protein concentrations were estimated as described above.

Two New Zealand White rabbits were inoculated with purified protein NC10-Ssp13 by Prof. A. Tait (Department of Veterinary Parasitology, University of Glasgow) to raise polyclonal sera against this recombinant antigen. The rabbit anti-

sera were then used in western blot analysis and IFAT as described in sections 4.2.3 and 4.2.16, respectively.

4.2.15.2. Preparation and purification of recombinant antigen from pGex Expression Vectors

Ligations were transformed into XL1-Blue competent cells, plated onto LB agar plates containing 100 μg/ml ampicillin and incubated overnight at 37°C. The presence of inserts was analysed by either restriction digestion of purified plasmid DNA or by hybridisation to colony lifts as described in section 4.2.15.1.

To analyse expression from the pGex constructs, the small scale culture method described in section 4.2.15.1 was adapted by altering the incubation period following protein induction. Thus, for pGex expression, cells were grown for 2 hours at 37°C after induction and the samples then processed for SDS-PAGE. The pGex 5x-1 or the pGex 5x-2 vector in XL1-Blue cells was used as a positive control.

Fusion proteins were purified on Glutathione-Sepharose 4B columns (Pharmacia) from lysates of *E. coli* by modifying the method of Smith and Johnson (1988). Overnight cultures of pGex transformed *E. coli* were used to inoculate 500 ml pre-warmed 2xYT (Sigma) medium supplemented with 0.2% glucose and 100 μg/ml ampicillin. This was incubated for 2-3 hours at 37°C. Induction with 2 mM IPTG was followed by incubation for a further 2-3 hours. Cultures were then harvested by centrifugation at 4,000 x g for 20 minutes at 4°C. Pellets were resuspended in 10 ml of cold PBS, 1% Triton X-100 (Sigma) containing protease inhibitors (10 μM 1,10 phenanthroline, 0.1 mM PMSF). The suspension was sonicated 9 times for 30 seconds on ice, at maximum amplitude of 6 microns, with a 30 second interval between each burst. The lysate was then centrifuged at 10,000 x g for 20 minutes at 4°C. The resulting supernatant was added to 2 ml Glutathione-Sepharose 4B, previously equilibrated in PBS, %1 Triton X-100. The mixture was stirred at room temperature for 45 minutes and loaded into a 1.6 cm diameter column. The column was washed with 50 ml PBS, 1% Triton X-100 and then with 50 ml PBS. Purified protein was eluted with 10 ml elution buffer (10 mM reduced glutathione, 50 mM Tris.HCl pH 8.0) in 1 ml fractions and analysed by 10% SDS-PAGE. The fractions containing purified protein were then dialysed against several

changes of PBS, pH 7.4, at 4°C. Concentrations of the eluted fusion proteins were estimated as described in section 4.2.15.1.

4.2.16. Indirect Fluorescent Antibody Test

An indirect immunofluorescent antibody test (IFAT) based on the method of Minami *et al.* (1983) was performed using isolated *T. annulata* infected cells as antigen. Cells in RPMI medium were harvested onto cytospin slides by spinning 100 µl of culture in a Shandon Cytospin at 240 x g for 5 minutes. When necessary, the density was adjusted by dilution of cells in RPMI medium. Slides were then air dried, and fixed by immersion in acetone at -20°C for 15 minutes. 20 µl of the first antibody, diluted in RPMI medium at dilution of 1 in 100-400, was spotted onto the fixed cells and the slides placed in a humidified box for 30 minutes at room temperature. Unbound antibodies were removed by 2 x 5 minute washes in PBS. The slides were then air dried before the addition of 20 µl fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG raised in goat (Sigma; F-0511), at a dilution of 1:100 in complete RPMI culture medium. The slide was again incubated in a humidified box for 30 minutes at room temperature, washed in PBS and counter-stained with Evans Blue (0.1% in PBS) for 5 minutes. The slide was mounted with a few drops of 50% glycerol/H_2O containing 2.5% w/v 1,4 diazabicycloctane (DABCO), pH 8.0. A Leitz Ortholux II fluorescence microscope and an Orthomat-W Camera attachment were used to record the results.

4.3. RESULTS

4.3.1. Identification of Macroschizont Antigens by Western Blot Analysis

In order to define macroschizont antigens that could be candidates for use in a diagnostic ELISA western blot analysis was carried out using antisera from animals immunised with attenuated cell lines. Serum samples tested against protein extracts from *T. annulata* Ankara D7 macroschizont infected cells and *T. annulata* Ankara piroplasms are indicated in Table 4.2. BL20 extracts were used as negative controls. Western blot conditions were optimised on the basis of serum, antigen and conjugate concentrations (see section 4.2.3). However, non-specific background staining observed with some of the pre-infection serum samples and with the uninfected BL20 control could not be totally eliminated (Figure 4.1).

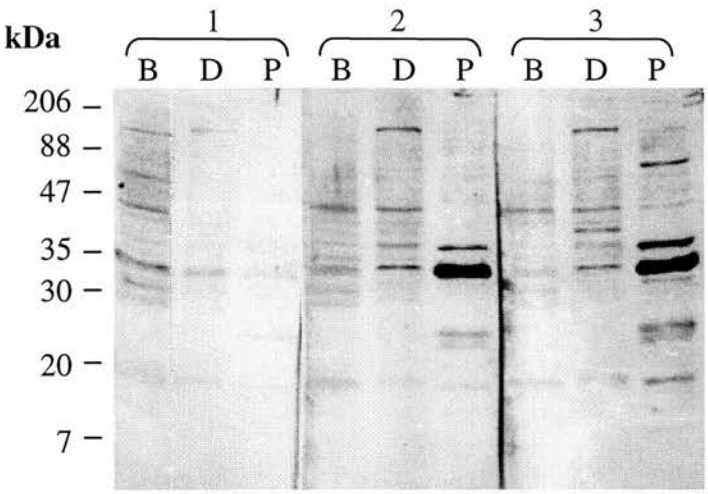
Serum samples obtained from animals immunised with attenuated cell lines, except *T. annulata* Razi, strongly recognised piroplasm antigens, especially those of estimated molecular masses of 30-37 kDa and 80 kDa. Figure 4.1 represents typical results of the western blot analysis performed with sera indicated in Table 4.2. In general, there was no strong recognition of schizont antigens by these serum samples. However, a specific set of schizont antigens as well as piroplasm antigens was recognised using sera from animals immunised either with non-attenuated cell lines or sporozoites (data not shown). Sera from calves N57, N58 and 155 recognised three major schizont specific antigens. Molecular masses of these polypeptides were estimated to be approximately 130 kDa and 40 kDa (Figure 4.1 blot 11). These polypeptides were absent in piroplasm extracts and were not detected with pre-immune bovine sera.

Since there were no serum samples from calves infected with high passage attenuated cell lines that specifically recognised macroschizont antigens, a second approach was chosen to define macroschizont specific antigens. This approach, which was based on a strategy of blocking the recognition of piroplasm antigens, was initially tested on western blots using a rabbit antiserum (C9m) raised against *T. annulata* merozoites. Thus, as merozoites and piroplasms are antigenically very similar (Glascodine *et al.*, 1990), preincubation of blots with the rabbit antiserum, followed by incubation with bovine immune serum and an anti-bovine immune

Table 4.2. Details of serum samples used to probe western blots using the *T. annulata* Ankara D7 macroschizont infected cell line, *T. annulata* Ankara piroplasms and the uninfected BL20 cell line as antigens. All these sera were obtained from calves experimentally immunised and challenged with a variety of *T. annulata* stocks.

Calf No	Primary immunisation with *T. annulata*	Challenge with *T. annulata*	Serum tested	
			days after immunisation	days after challenge
26A, 32A, 54C, 891A	Ankara/Pendik (passage 317)	Gharb (sporozoite stabilate)	0, 28, 56, 84	28
7, 3642	Diyarbakir (passage 467)	Gharb (sporozoite stabilate)	0, 28, 56	28
56V, 749, 750	Ode (passage 406)		0, 28	
15Z,118V, 16Z	Tova (passage 2-3)		0, 44	
18P, 19P, 23	Razi (passage 450)		0, 35	
N57, N58	Gharb (passage 4)	Gharb (passage 8)	0, 28	30
17, 32C, 892A, 22A	Ankara (passage 5)	Gharb (sporozoite stabilate)	0, 28, 56, 84	28
155	Ankara (sporozoite stabilate)	Ankara (sporozoite stabilate)		28

Figure 4.1. Western blot analysis of serum samples from animals immunised with low and high passage cell lines. Calves 26A, 32A and 891A were immunised with the *T. annulata* Ankara/Pendik (passage 317) cell line, calf 58 was immunised with *T. annulata* Gharb (passage 5). Blots 1-3: calf 26A days 0, 28, 56; blots 4-6: calf 32A days 0, 28, 56; blots 7-9 calf 891A days 0, 28, 56; blots 10-11: calf N58 days 0, 28.
B: BL20 cell line; D: the clonal *T. annulata* Ankara D7 cell line; P: *T. annulata* Ankara piroplasm. 20 µl of BL20 and D7 and 5 µl of piroplasm protein extracts loaded onto each track. The positions of size markers are shown in each panel.


kDa
1
2
3
B D P B D P B D P
206
88
47
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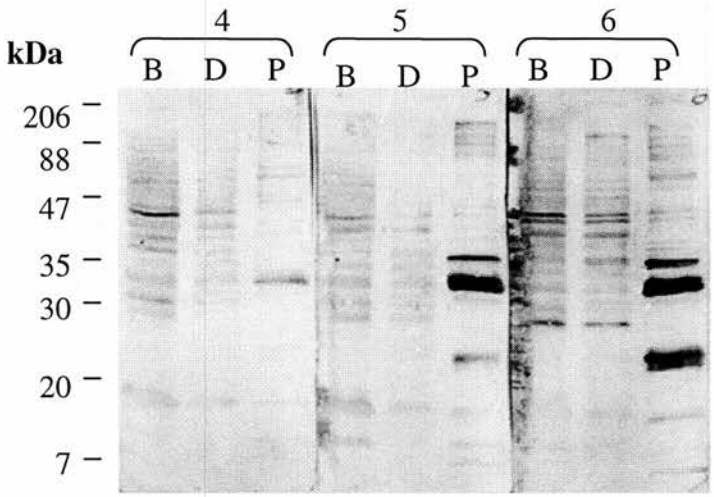
30
20
7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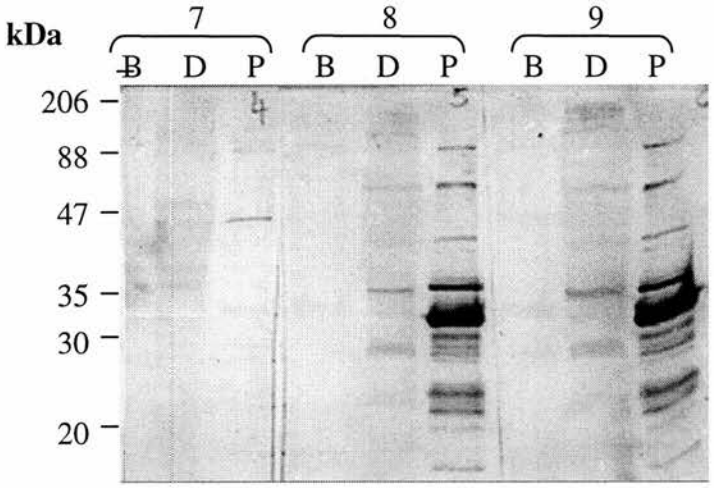
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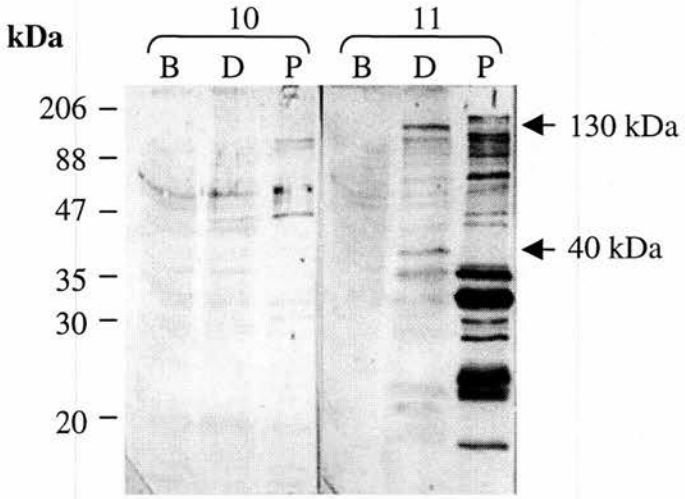
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130 kDa
40 kDa

conjugate, would result in a reduction of piroplasm antigens detected but allow recognition of antigens specific to the schizont stage. If successful, this method could then be used to screen a genomic expression library. Serum samples from calves N57, N58 and 155 were tested using western blots of BL-20, *T. annulata* Ankara D7 and piroplasm extracts. While the pre-incubation of these blots with several different dilutions (1:10 – 1:500) of C9m rabbit serum eliminated only some of the piroplasm bands or reduced to intensity of their reaction (Figure 4.2), the immune bovine serum still reacted with several piroplasm antigens.

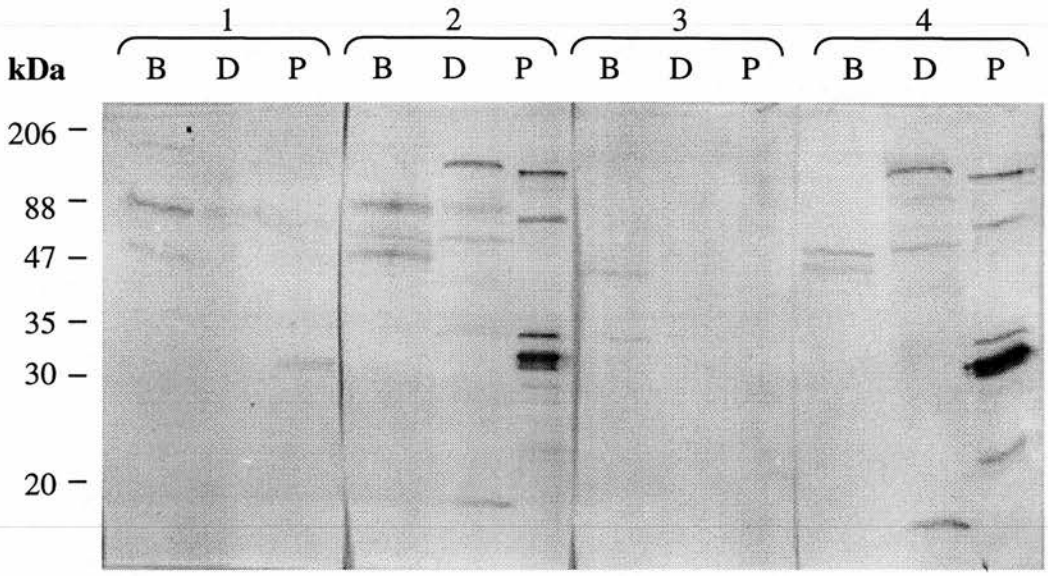
Another approach used to eliminate the recognition of piroplasm antigens was blocking of antibodies against piroplasms in immune sera using piroplasm lysate. After incubations of serum samples with piroplasm lysate, only a limited number of piroplasm antigens were detected at high molecular mass (around 120 kDa in size) (Figure 4.3). Most of the reactions with macroschizont antigens were also reduced by this methodology. Only one macroschizont antigen at about 40 kDa was recognised strongly (Figure 4.3). This polypeptide was previously observed and shown to exhibit size polymorphisms (Shiels *et al.*, 1994; Shiels per. comm.). On the basis of these results, the immunoabsorption technique using piroplasm lysate was used to screen a genomic expression library in order to isolate the gene encoding the 40 kDa macroschizont specific antigen.

4.3.2. Attempted Cloning of the Gene Encoding the 40 kDa Macroschizont Antigen

An available λgt11 *T. annulata* genomic expression library was screened with bovine serum-155 following blocking of piroplasm antibodies in the serum by immunoabsorption with piroplasm lysate (see section 4.2.5). No positive plaques were identified with this bovine serum (data not shown). The screening experiment was not repeated with additional antisera or altered incubation times because of time constraints. Since the 40 kDa protein shows size polymorphism and it is immunodominant, it is possible that this protein could be related to the QP/PIM gene of *T. parva*. If this were the case, isolating the homologue of this gene by screening a cDNA or genomic DNA library with a QP gene probe would provide an alternative cloning strategy.

Figure 4.2. Western blot analysis of immune bovine serum after blots were blocked with rabbit anti-merozoite serum. Blots 1 and 2: Calf N58 day 0 and day 58, incubated with rabbit anti-merozoite serum; blots 3 and 4: Calf N58 day 0 and day 58, control blots. B: BL20 cell line; D: *T. annulata* Ankara D7 clonal cell line; P: *T. annulata* Ankara piroplasm. 20 µl of BL20 and D7 and 5 µl of piroplasm protein extracts loaded onto each lane. The numbers on the left indicate the molecular weights of the marker proteins.

Figure 4.3. Western blot analysis of serum samples after immunoprecipitation of piroplasm antibodies using piroplasm extract. Blot 1 was probed with immune bovine serum (calf 155) after elimination of piroplasm antigens by immunoprecipitation. Blot 2 was probed with immune bovine serum (calf 155) as a control. B: BL20 cell line; D: *T. annulata* Ankara D7 clonal cell line; P: *T. annulata* Ankara piroplasm. 20 µl of BL20 and D7 and 5 µl of piroplasm protein extracts loaded onto each lane. The numbers on the left indicate the molecular weights of the marker proteins.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B D P B D P B D P B D P
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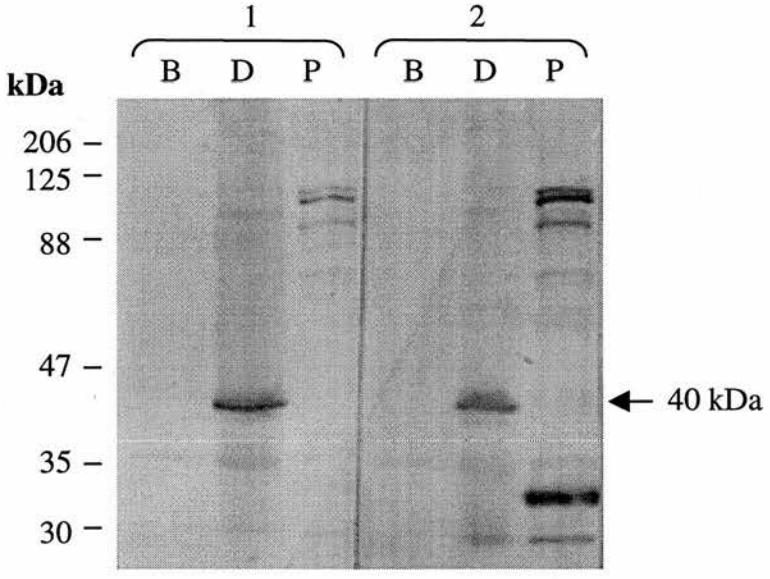
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B D P B D P
206
125
88
47
35
30
40 kDa

4.3.3. Establishing the Existence of a QP Homologue in *T. annulata*

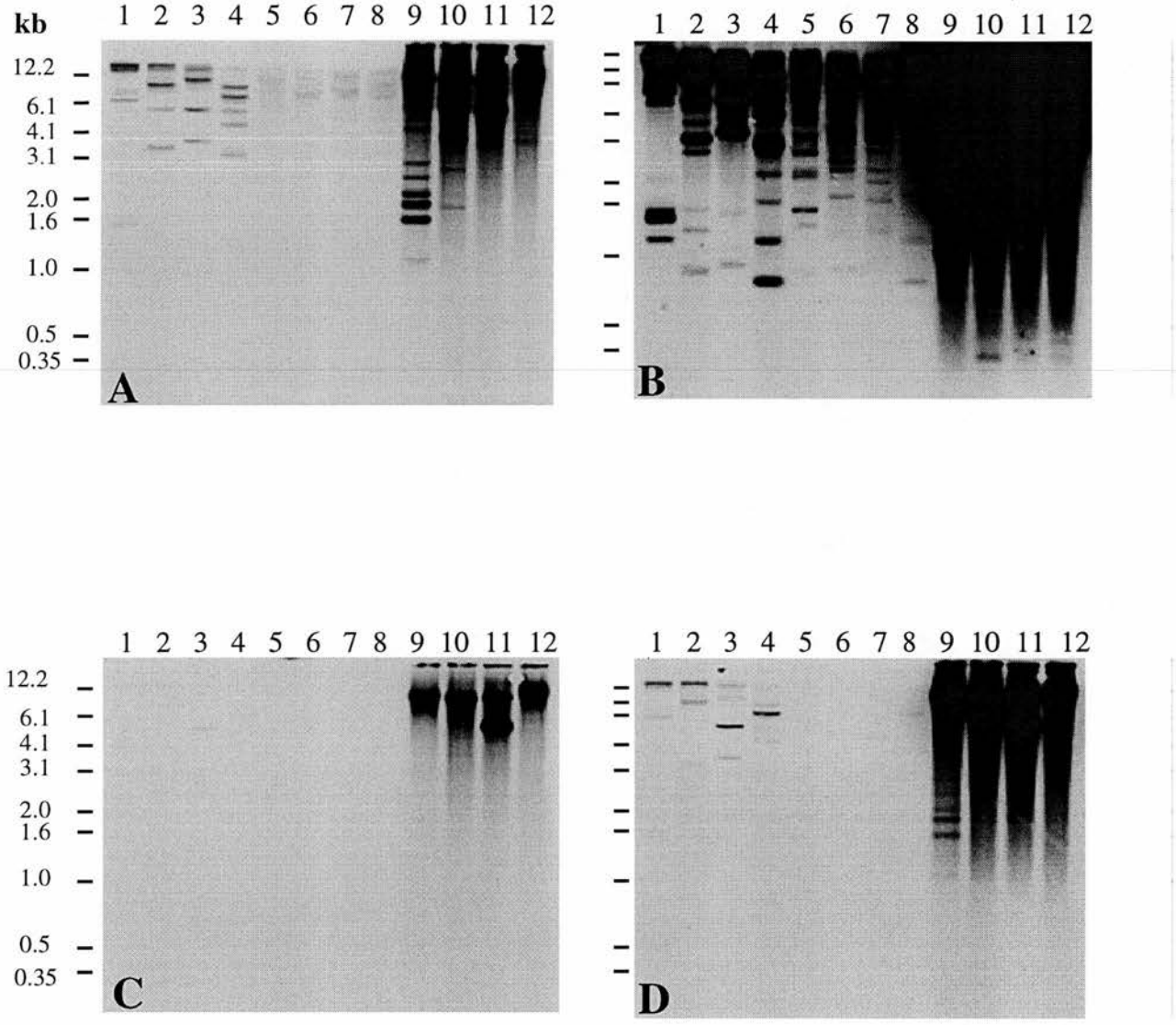
4.3.3.1. Southern blot profile of QP/PIM

Southern blot analysis was carried out to test for the presence of a *T. parva* QP protein gene homologue in the *T. annulata* genome. The genomic DNA from the *T. annulata* Ankara D7 cell line, *T. annulata* Ankara piroplasms and *T. parva* Muguga piroplasms was digested with *Bam*HI, *Eco*RI, *Hind*III and *Xba*I. Hybridisation of the Southern blot was carried out at 55°C using the QP cDNA probe. The blot was washed with 1 x SSC, 5% SDS at 55°C and exposed to an X-ray film for 2.5 hour and overnight. The blot was then washed at 60°C and 65°C and exposed to film as above.

At low stringency (55°C), many DNA restriction fragments were detected on *T. parva* genomic DNA (Figure 4.4 lanes 1-4) indicating the presence of many QP related genes in the *T. parva* Muguga genome. Following high stringency washes, there was only one band visible in all the digests, except for *Hind*III as reported previously (Baylis *et al.*, 1993; Toye *et al.*, 1995a) (Figure 4.4C). However, longer exposure revealed several bands ranging from 0.5 kb to >12 kb (Figure 4.4D).

The QP cDNA probe identified several restriction DNA fragments ranging from 0.5 kb to >12 kb in *T. annulata* Ankara, using both DNA from macroschizont infected cells (representing a single parasite genotype) and piroplasms (Figure 4.4A and B, lanes 5-12) at low stringency. Following high stringency washes, many of the bands disappeared on *T. annulata* DNA. However, some of the bands between 3 kb and >12 kb were still visible on *T. annulata* Ankara piroplasm DNA following overnight exposure (Figure 4.4C and D, lanes 9-12). The bands in lanes representing the macroschizont DNA (lanes 5-8) were much fainter. This result is to be expected, since the relative amount of parasite DNA loaded was significantly less in macroschizont infected cell DNA than piroplasm DNA. These results indicated that there are likely to be at least several genes in the *T. annulata* genome with homology to the QP probe.

Figure 4.4. Southern blot analysis of the gene encoding the QP protein. Genomic DNA was prepared from *T. annulata* Ankara piroplasms (lanes 1-4), *T. annulata* Ankara D7 macroschizont infected cell line (lanes 5-8) and *T. parva* Muguga piroplasms (lanes 9-12). Genomic DNA was digested with *Bam*HI (lanes 1, 5, 9), *Eco*RI (lane 2, 6, 10), *Hind*III (lanes 3, 7, 11) and *Xba*I (lane 4, 8, 12). The blot was hybridised with the ^{32}P labelled QP probe at low stringency. Washes were performed first at 55°C (A and B) and then at 65°C (C and D). The blot was exposed either for 3 hours (A and C) or overnight (B and D). Sizes of the DNA markers are shown in kilobase pairs (kb).


kb
1 2 3 4 5 6 7 8 9 10 11 12
12.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.35
A
B
C
D

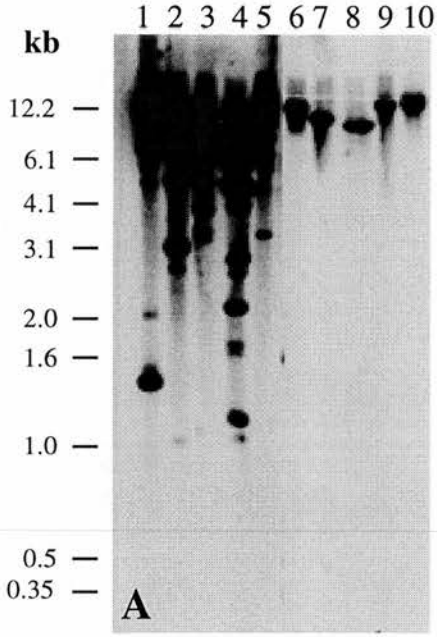
4.3.3.2. Analysis of the copy number of QP related genes in T. annulata

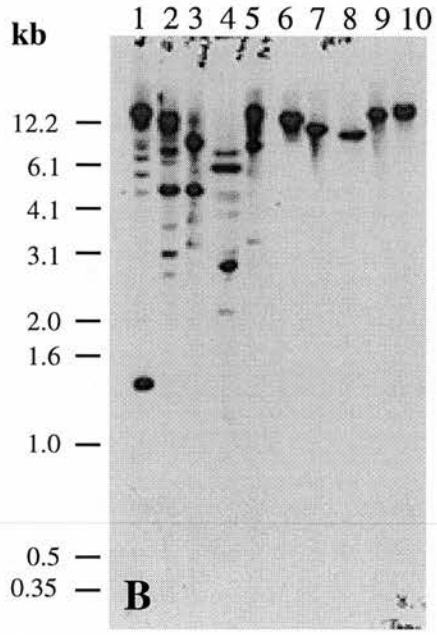
The gene that encodes the QP protein has been reported to be single copy in the *T. parva* genome (Baylis *et al.*, 1993). Since Southern blot analysis of the *T. parva* Muguga piroplasm DNA revealed many bands at low stringency, the hybridisation pattern of the QP probe of *T. annulata* genomic DNA was compared with that of a single copy gene, Tams-1. This gene encodes the 30 kDa merozoite/piroplasm surface antigen and was shown to be a single copy gene in *T. annulata* (Shiels *et al.*, 1995). DNA from *T. annulata* Gharb piroplasms and the macroschizont infected cloned cell line (D7) were digested with several restriction enzymes (*Bam*HI, *Eco*RI, *Hind*III, *Xba*I and *Pst*I). The hybridisation of the Southern blot was carried out at 55°C for both probes. The blots were washed first at 55°C, then 60°C and finally 65°C. The QP probe detected several restriction fragments with piroplasm DNA at low stringency as described above, although there was a decrease in the number of bands with increasing stringency using the QP probe. In contrast, the Tams-1 probe hybridised to a single restriction fragment for each of the digests (Figure 4.5) under low, medium and high stringency conditions. It was previously demonstrated that Tams-1 probe detects a single band with DNA of D7 cell line indicating that the Tams-1 is a single copy gene (Shiels *et al.*, 1995). Figure 4.5D shows the results of Southern blotting after the 60°C washes using DNA from the D7 cell line. Two strong bands were detected for each restriction enzyme digestion indicating that the *T. annulata* genome may contain more than one gene that is closely related to the QP probe.

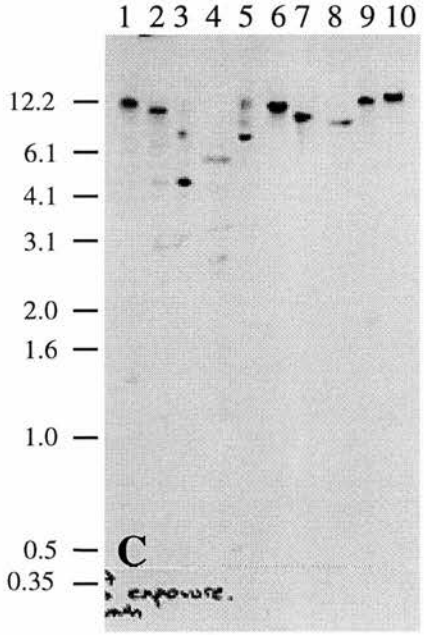
4.3.4. Cloning and Characterization of the QP Homologue of *T. annulata*

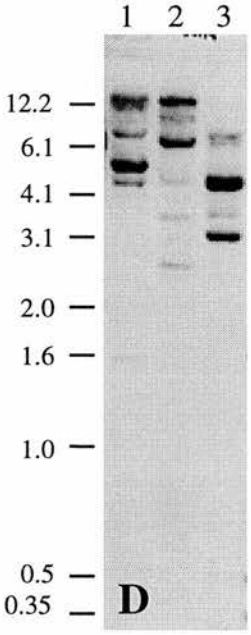
A λ ZAP cDNA library of the *T. annulata* Ankara macroschizont infected cell line D7 was screened in order to isolate the *T. annulata* homologue of the QP/PIM gene using a cDNA probe. Hybridisation of the library with a 1.7 kb *Eco*RI gel-purified QP gene fragment was performed at 55°C and the filters were washed 3 times at 55°C with 1 x SSC, 5% SDS for 20 minutes. A total of 64 positive plaques were picked from the first round of library screening. 24 positive plaques, randomly selected, were taken to subsequent rounds of screening. Of these, 14 consistently hybridised with the probe and were purified to homogeneity.

Figure 4.5. Analysis of the copy number of QP related genes in *T. annulata*.
A-C) Genomic DNA was prepared from *T. annulata* Gharp piroplasms. DNA was digested with *Bam*HI (lanes 1, 6), *Eco*RI (lanes 2, 7), *Hind*III (lanes 3, 8) and *Xba*I (lane 4, 9) and *Pst*I (lanes 5, 10). The blots were hybridised with the ^{32}P labelled QP probe (lanes 1-5) and Tams-1 probe (lane 6-10) at low stringency. Washes were performed first at 55°C (A), then at 60°C (B) and 65°C (C).
D) Genomic DNA from the clonal *T. annulata* Ankara D7 cell line was digested with *Bam*HI (lane 1), *Eco*RI (lane 2) and *Hind*III (lane 3). The blot was hybridised with the ^{32}P labelled QP probe (lanes 1-5) at 55°C and washed at 60°C. Sizes of the DNA markers are shown in kilobase pairs (kb).


1 2 3 4 5 6 7 8 9 10
kb
12.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.35
A


1 2 3 4 5 6 7 8 9 10
kb
12.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.35
B


1 2 3 4 5 6 7 8 9 10
12.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.35
C


1 2 3
12.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.35
D

The inserts of λ ZAP phage clones were then excised into the pBluescript plasmid (see section 4.2.8) and the size of the inserts was estimated by restriction digestion with *Sma*I and *Kpn*I and agarose gel electrophoresis. Southern blot analysis of the gel with the QP probe confirmed that the inserts were related to the QP gene (data not shown).

Initial characterization of the 14 clones was carried out by sequence analysis of the 5' and 3' ends of each insert. The λ ZAP cDNA library was constructed so that the 5' end of the *Theileria* cDNA inserts were ligated into the *Eco*RI site and the 3' end ligated into the *Xho*I site of the vector. This orientation of the cloned cDNA inserts was confirmed as tracks of poly A sequence were seen at the 3' end of each clone. Comparison of the 14 nucleotide sequence with each other resulted in the establishment of four distinct groups of cDNA clones (Table 4.3).

Group 1 (called NC1) consisted of 9 clones. One of the clones in this group (NC11) has an additional 33 bp insert at the position of 1804 on the NC1 consensus sequence that did not align with the sequences of the other clones in this group. The nucleotides 5' [GT.....AG] 3', flanking this insertion, suggested that it represents an unspliced intron.

Group 2 (called NC10) consisted of three clones, which were identical at the 3' end. Although the size of the insert of clone "NC5" was smaller than the remaining two clones in this group, the 5' end of the sequence did not align with that of the other two clones.

Group 3 (called NC2) and Group 4 (called NC9) consisted of only one clone each.

The sequence obtained for the 5' and 3' end of each clone was compared with the sequences in the EMBL database. The sequences from groups 1, 2 and 3 did not have any significant homology to any known gene. However, the sequence of NC9 had a high homology score with a bovine ribosomal protein gene (97.2% identity over 686 bp).

4.3.5. Genomic Arrangement of Genes Isolated from the cDNA Library

To determine the organization of the genes isolated from the λ ZAP cDNA library, Southern blot analysis was carried out using genomic DNA from both

Table 4.3. Groups of cDNA clones isolated using QP protein probe from λ ZAP cDNA library of the *T. annulata* Ankara D7 macroschizont infected cell line. The sequence analysis of the 5' and 3' end of each clone was carried out using T7 and M13 primers.

Group	cDNA clone	Size/Kb
Group 1	NC1, NC6, NC16, NC19, NC21, NC22, NC23	3.4
	NC11	1.8
	NC20	5.5
Group 2	NC5	3.0
	NC10, C15	3.5
Group 3	NC2	3.0
Group 4	NC9	2.2

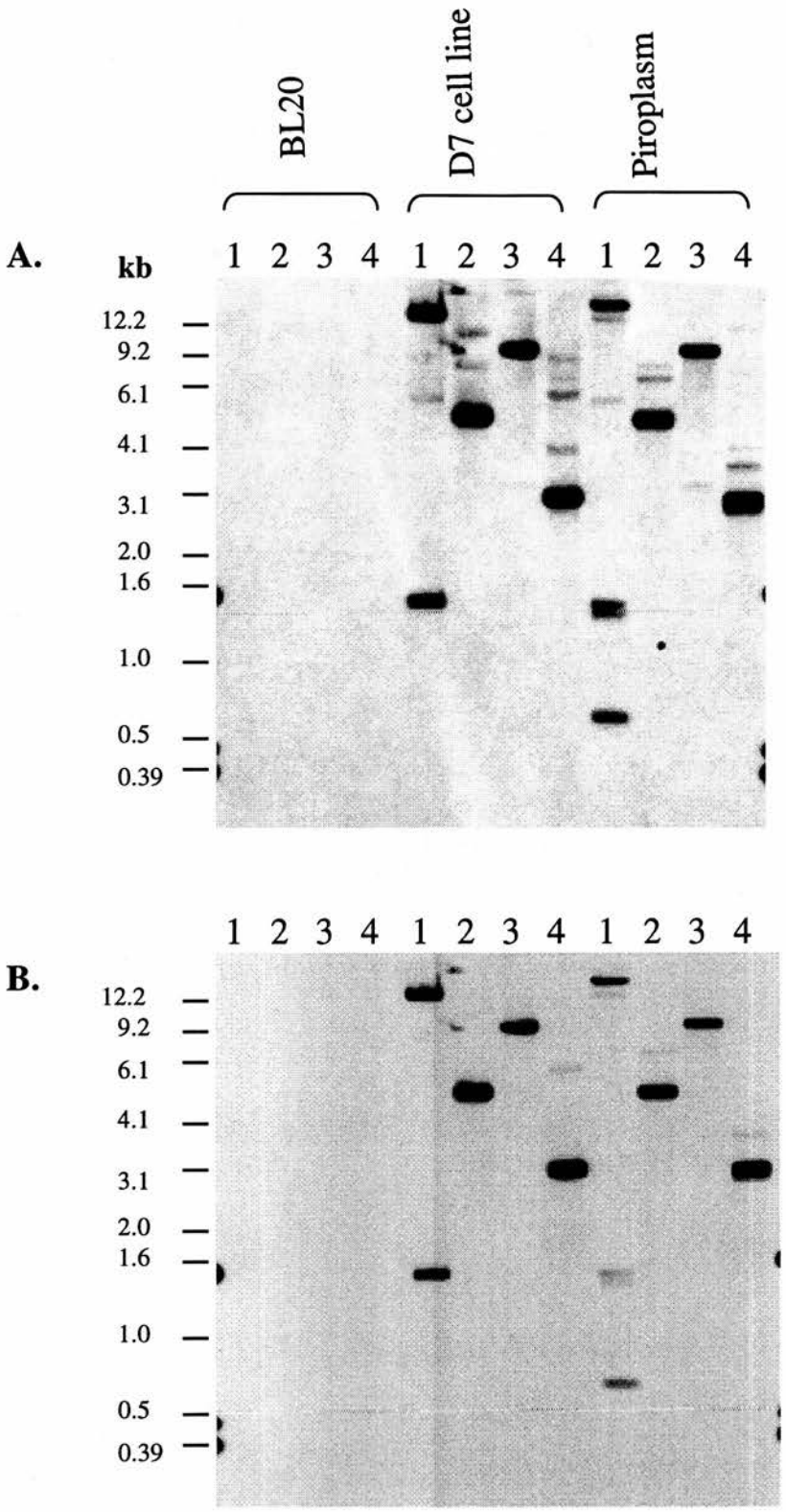
piroplasms and the D7 cell line. Bovine DNA from the uninfected cell line BL20 was used as a control. The DNA inserts of plasmid clones, NC1, NC10 and NC2, were released with *Sma*I and *Kpn*I and used to probe Southern blots at 55°C followed by washing at 55°C (low stringency) and then at 65°C (high stringency).

4.3.5.1. Group 1 (Clone NC1)

The cDNA clone NC1 hybridised to several restriction fragments in each of the digests of both macroschizont and piroplasm DNA at low stringency. The molecular sizes of these fragments ranged between 0.7 kb and >12 kb (Figure 4.6A). However, one of the bands in each digest was stronger than the other bands, except in the *Bam*HI digest where two strong bands were observed. A single band remained with digests of the macroschizont DNA following washing at 65°C, except for the *Bam*HI digest (Figure 4.6B).

The hybridisation pattern detected by clone NC1 differed between the *Bam*HI digestion of macroschizont and piroplasm DNA. Four DNA fragments were detected in the piroplasm DNA, *i.e.* a 0.7 kb fragment, a doublet at approximately 1.6 kb and a large fragment (>12 kb). The 0.7 kb band was not present in the digest of macroschizont infected cell DNA and there was only a strong single band at 1.6 kb as well as a large band (>12 kb) which was slightly smaller than the equivalent band observed in the piroplasm track. Sequence analysis of NC1 clone revealed the presence of an internal *Bam*HI site in the cDNA clone, indicating that the gene represented by clone NC1 is likely to be single copy gene. Differences in the hybridisation pattern between macroschizont and piroplasm DNA is likely to be due to restriction fragment length polymorphism (RFLP) between different genotypes of the parasite population. The results indicated that the major genotype present in the piroplasms differs from the D7 cell line with regard to the *Bam*HI RFLP while the other restriction sites are conserved between D7 and major piroplasm genotypes. No hybridisation was detected with bovine DNA under low or high stringency conditions, indicating that the cloned insert represents a parasite gene and that it is not of bovine origin.

Figure 4.6. Southern blot analysis of the *T. annulata* NC1 clone. Genomic DNA was prepared from the uninfected BL20 cell line, the clonal *T. annulata* Ankara D7 cell line and *T. annulata* Ankara piroplasms. DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3) and *Xba*I (lane 4). The blot was hybridised with the ^{32}P labelled NC1 cDNA probe under low stringency condition. Washes were performed first at 55°C (A) and then at 65°C (B). Sizes of the DNA markers are shown in kilobase pairs (kb).

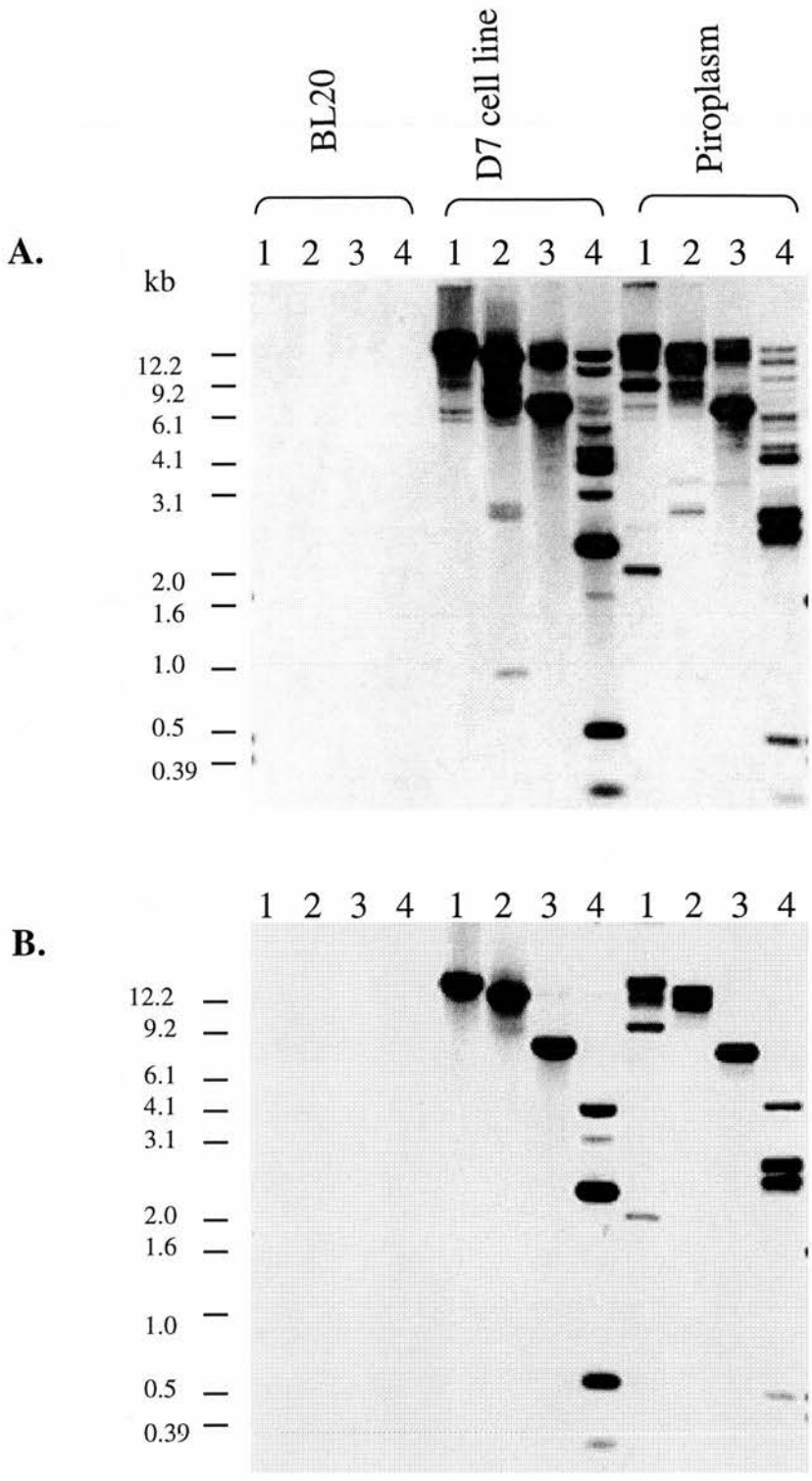

BL20
D7 cell line
Piroplasm
A.
kb
1 2 3 4 1 2 3 4 1 2 3 4
12.2
9.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.39
B.
1 2 3 4 1 2 3 4 1 2 3 4
12.2
9.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.39

4.3.5.2. Group 2 (Clone NC10)

The cDNA clone NC10 hybridised to several restriction fragments in digests of both macroschizont and piroplasm DNA at low stringency. The sizes of these fragments ranged from 0.2 kb to >12 kb (Figure 4.7A). For DNA of the macroschizont cell line, a single restriction fragment remained visible following washes at 65°C, except for the *Xba*I digest (Figure 4.7B). Three strong and two weak bands were observed with the *Xba*I digest. Sizes of these fragments were approximately 0.2 kb, 0.55 kb, 2.5 kb, 3.1 kb and 4.1 kb. Sequence analysis demonstrated that there are three internal *Xba*I sites within the region covered by the cDNA probe. These *Xba*I sites were located at nucleotides 2424, 4142 and 4349 of the NC10 consensus sequence (data not shown). Data obtained from the genomic sequence showed that there was another *Xba*I site located at nucleotide 1910. On the basis of this information, four restriction fragments, 207 bp, 514 bp, 1718 bp in size and another fragment with a minimum size of 1 kb, would be expected. Therefore, the 0.2, 0.55 and 4.1 kb fragments detected by Southern blotting matched the estimated sizes of the fragments determined by sequence analysis. However, a strong band at about 2.5 kb and a faint band at about 3.1 kb were larger than expected. The PCR amplification and preliminary sequence analysis of part of the 1.7 kb fragment revealed an intron of approximately 750 bp (data not shown), thus increasing the size of this *Xba*I RFLP fragment from 1718 bp about 2.5 kb. Therefore, the 3.1 kb faint band could be the result of a partial digest of the 2.5 kb and the adjacent 514 bp fragments. These results would suggest that the NC10 gene is a single copy gene. Alternatively, the 3.1 kb band could be due to the presence of another NC10 gene in the *T. annulata* genome or a gene that is closely related to NC10.

The analysis of the piroplasm DNA also detected several bands under high stringency conditions in all digests except *Hind*III. One of the bands in each digest of piroplasm DNA was at the same size as that observed in macroschizont DNA. This indicates that it is likely that for these enzymes RFLPs exist in the major parasite populations represented in the piroplasm DNA with *Bam*HI, *Eco*RI and *Xba*I. No bands were detected with bovine DNA under both low and high stringency conditions indicating that the gene is of parasite origin.

Figure 4.7. Southern blot analysis of the *T. annulata* NC10 clone. Genomic DNA was prepared from the uninfected BL20 cell line, the clonal *T. annulata* Ankara D7 cell line and *T. annulata* Ankara piroplasms. DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3) and *Xba*I (lane 4). The blot was hybridised with the ^{32}P labelled NC10 cDNA probe under low stringency condition. Washes were performed first at 55°C (A) and then at 65°C (B). Sizes of the DNA markers are shown in kilobase pairs (kb).


A.
BL20
D7 cell line
Piroplasm
1 2 3 4 1 2 3 4 1 2 3 4
kb
12.2
9.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.39
B.
1 2 3 4 1 2 3 4 1 2 3 4
12.2
9.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.39

4.3.5.3. Group 3 (Clone NC2)

At low stringency the cDNA clone NC2 hybridised to many restriction fragments in digests of both macroschizont and piroplasm DNA (Figure 4.8A). The size of these bands ranged from 1.2 kb to >12 kb. Following high stringency washes at 65°C, many of the bands were removed leaving only a single restriction fragment for the *Bam*HI digest (Figure 4.8B). The presence of internal restriction enzyme sites for *Eco*RI, *Hind*III and *Xba*I was confirmed by digesting the NC2 plasmid DNA with these enzymes. On the basis of the *Bam*HI digest and the presence of internal sites in clone NC2 it is likely to be a single copy gene with related DNA sequences in the genome. On the other hand several bands observed with each digestion could be due to the presence of more than one NC2 gene in the genome.

4.3.6. Full Length Nucleotide Sequences

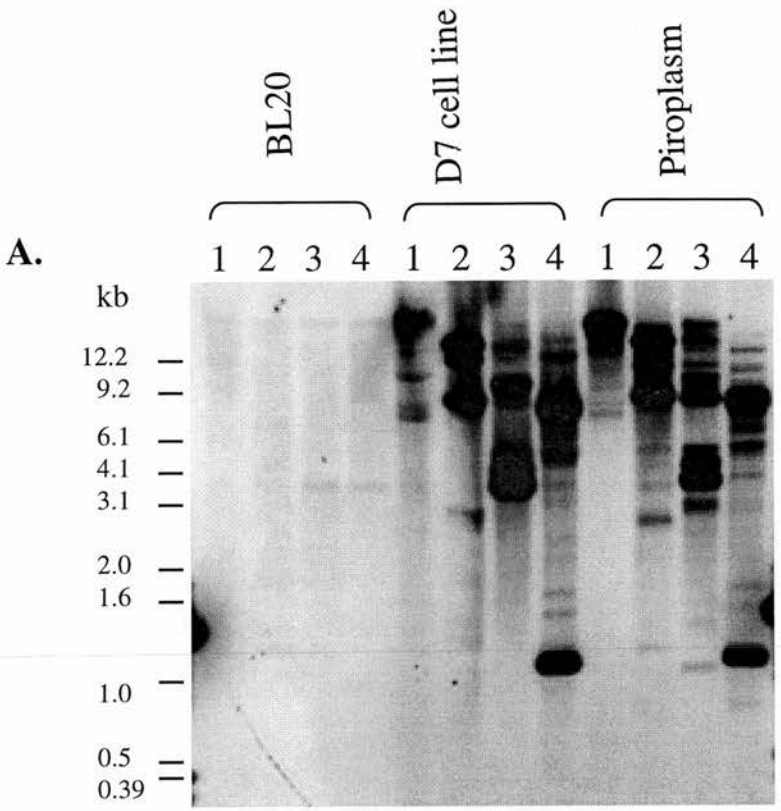
One representative clone, with the largest insert, from each group was chosen and fully sequenced on both strands. Clone NC1 was chosen from Group 1 and clone NC10 from Group 2. It was not possible to sequence the clone in Group 3 because of time constraints.

4.3.6.1. Nucleotide sequence determination of clone NC1

The cDNA clone NC1 had an insert of about 3.2-3.4 kb. Exo III deletions of this insert were generated using the methodology outlined in section 4.2.12. For the reverse orientation of the insert, there were no appropriate restriction enzymes to generate the deletions. In order to create appropriate sites, the insert was released from the pBluescript vector, ligated into pGem 7zf(+) and Exo III deletions were made.

Sequence analysis demonstrated that the insert is 3192 bp long, has a single open reading frame (ORF) of 2280 bp that starts from nucleotide 691 and extends to a stop codon ending at nucleotide 2970 (Figure 4.9A). The cDNA encodes a protein of 760 amino acids (Figure 4.9B). The ATG start codon at the beginning of the ORF (at the position 691) is the 17th 'ATG' in the cDNA.

Figure 4.8. Southern blot analysis of the *T. annulata* NC2 clone. Genomic DNA was prepared from the uninfected BL20 cell line, the clonal *T. annulata* Ankara D7 cell line and *T. annulata* Ankara piroplasms. The DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3) and *Xba*I (lane 4). The blot was hybridised with the ^{32}P labelled NC2 cDNA probe under low stringency condition at 55°C. Washes were performed first at 55°C (A) and then at 65°C (B). Sizes of the DNA markers are shown in kilobase pairs (kb).


A.
BL20
D7 cell line
Piroplasm
1 2 3 4 1 2 3 4 1 2 3 4
kb
12.2
9.2
6.1
4.1
3.1
2.0
1.6
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0.39

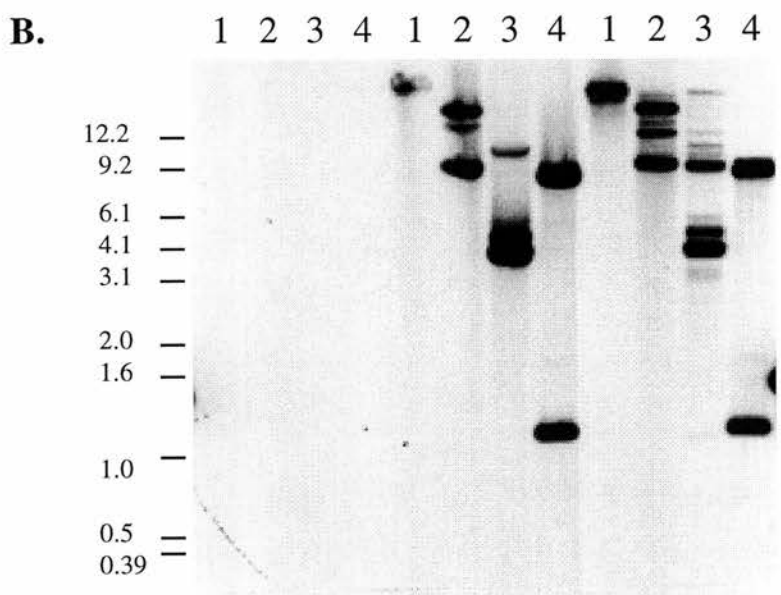

B.
1 2 3 4 1 2 3 4 1 2 3 4
12.2
9.2
6.1
4.1
3.1
2.0
1.6
1.0
0.5
0.39

Figure 4.9A. The nucleotide sequence of the NC1 gene. The numbers represent the sequence position with reference to the most 5' base. ATG start and TAA stop codons are underlined.

```
   1  GGCACGAGTA TTGTTACTGT CGATTGTTCT ATTATCTATC CTAAAAATAT
  51  CGCATGCTTC CGATAAAGAT CCACAGAAGG ATCCACAAAA AAAGGATGTT
 101  GATCCTTTAC ACCATGTAAA TGTTCATTTA AGACTTGTGC AAAGGTTATT
 151  TTCAAGGGAC TCTACTCAAT TACCTGATCA ACCTCAGCCT GAGCCTATTG
 201  GTGAACCTGA ATATGAGGAA TATGAGGAGG AATCGGACGA TGAAGAACTT
 251  GCAAGAAGTA TACTCGAAGA AAATGAGGAA CGGAGCAAGG AAATACAAAA
 301  ATGCAAGATC CAAAGTCTTG ACTTTTCAGA ACAAGATCTT GTAATGATGG
 351  TTATAGAAGA AATGGAATCT AAATCAGATG ATGAAGGGGA AGAAGGAAGT
 401  GATGAAGGTC ATCATGAGCC AGATCAACAG GAGAAAGAGG GAAAAAAGGA
 451  AGAAACAAAG AAACGAAAGA AAGTAGACGA TACTGATGAT GAAGAACAAA
 501  AGGAAACCGA AAAAAAGAAG AAACCTAAAC ATCAAGATAA ACCTGAGGGG
 551  TACCCCAAAG ACCTACACCG AAACCTAGAA CTAAACCAGA TACCAAGCCG
 601  AAACCATCAC CAATACCTAA GCCTAGAACT AAACCAGTTA CGATTGAAAA
 651  ACCATCACCT ACACCCAAAC CTAGAACTAA ACCAGGTACG ATGGAAACCC
 701  CGTCACCTAA GGCTGTGCCA CCTCCAAAAC CTAAACGCAC GAAAAAACCA
 751  TTAGCTCAAA CTGCACCTGT GGGATCTGAG GAAAAAGAAG ATAAGAAGGT
 801  TGAGGATACT GAACCAAAAC CTCAGGAACC TATAACTGAA ACAAAACCTG
 851  AACCAGAGCC ACAACAACCG CAACCGATAA CTGAACCAAT AACTGAACCA
 901  CAACCAAAGG AACCTGAACC TGAAGAACCT AAAGAACAAT CAATGCCAGA
 951  ATCTGGACCT GAAGGACCTA AGGAGACAGA AGATAAAGAT GCAACGGGAC
1001  CAGAAGAGCC TAAGCAGACA GAGCCTGAAC ATGAAAAACC AAAGGAACAA
1051  GATGATACTG AACCAAAACC TGATACAGAG CATGAACATG AAAAACATGA
1101  CTCACAAGAA GATAAAGATA CCAAGGAACG TCAGGAACAA GAGGATTCTG
1151  ATAAGAAAGA TGATTCTGTT CCAGAACCAG AACCAGAAGA ACCCAAGGAT
1201  ATTGACCATG TAGATGTGGA TGAACCAGAA CAAGAGGAAC AAGAGGATTC
1251  TGATAAGAAA GAAGATGAAA CTGAGGATAA ACATGAACCT CCAAAGGAAC
1301  AAGAAGATAT GGAGGCTGGG GATACTGAAC AAGAACCACA ACCACAACCA
1351  GAAGGACAGG AGGAAACTGA ACCTGAAGTA TCAAAGGATA CTGAACCAAA
1401  ATCAGAACCT ATACAAGATC CTATACCTGA CACAAAACAT GAAGATGAAC
1451  AGGAACAAGA GGATTCTGAT AAAGAAAAAC CTGATGCTGT CCCAGAAGAG
1501  CCTAAGGATA CTGACCATAA ACAAGAATCA GAAGAAGACA AAGATACCAA
1551  GGAACCAGAA GAGCCTACTC AACCTACTGA ACCTGAACCT GAACCTCAAC
1601  CTCAACAAGA GGTACAACCT GAGGAACCGA CGAAACCTCA GCCCCAGGAG
1651  ACTAAGGATC CTGATGATAA GCAGGAAGAA GAAAAGAAGC CTGATCCTGG
1701  GGAAGAAGAT CGAACTGATT TGGATAAAGA TGGACAAATG AAAACACCTG
1751  AGAAAATGAA ACCAGAGGAT ATTGGGGGAC CTGAATTTCC AGAGCCAGTA
1801  GATCAATCCA CGCCAAATAC TGTTAAACCC CCTATATTCG ATAAAGATCG
1851  CATAAAAACA CCTAGATTAG TCTTTTTGAC TGATGATGAT GACGGCGAAG
1901  GATCTGAACC TACTAAAAGA AGAAAGCCAT TCTTAATCTA TGATAGGCCT
1951  GGAAAAACTC CAAGAAGACC TAAGAAGAAA AAGATTAAAC AGCTTTTTGA
2001  GCATGATGAT GATGGTGACG GTGATGATGA AGATGAAGAT GAAGATGAAG
2051  AAAGTGATGA AGCTGAAGCT GAAATTCATA CAAGTGGAAC AAAAGATAGA
2101  AAAGATGACG GTGGAGATAA AGATGACGGA AGTGGAGGTT CAGGGGGTGC
2151  AGGCCCAACT CCTTCAACAG ATGAAAACAA CCAGGCTGGT GGTAGTGGAA
2201  GTGGAGAGGC AAAACATGAT GATAATGGGG ATTCAAATAC AGGAGGGTCC
2251  AAACAATCAG ACACGGGAAA TGGAGATGAT AGTAAAGATA AGGATGAAAA
2301  ACAGCAAGGT GAGGATGAAA AAGAAAAAGA AAAGGAGAAA AAGGAAGTTG
2351  AGCAAAAACT TCCTGTAACT GAAACACCCA GTACACCAGC AATATCAAAC
2401  CCAGTAACTA AAACACCTAA AACACCAAAA AAACGAAGAC GACCATCTAA
2451  GATACCTATT AAACAAACAA AAGTGAAACC AGCATCAAGA CCATCAACGA
2501  CGCCTGGTAA AGGACCATTT AAAAAGCCAA TAGTAACACC TGGTGGACCA
2551  TCAATAGTGA AACCAGTATC AAGACCATCA ACTTCAAGGC CATCATCTAC
2601  CACACCACCA ACTACAACAT CCGGAAGACA ACCTCCTAAA AGTGATGATC
2651  GAAGATCATC TCAACCACCA AGACCCCCGT TTGTTGTACC ATGGGCAACT
2701  AAAGAAATAC TCACTAAGGG TGCAACAAAA ACGCCACAAC AAGAACAAGG
2751  GACACAATTC TCTACAGAGG ATAGAACAAG AGAACAAGGA AAAAGGGCAG
2801  GAGGTGAAAC AAGGCCTGAT ACATCAAAAG GAGCAGTAGG TGAAGGAAGA
2851  TCTAGAAGAT CACAAAGGTT ATCAGCTAGA GGAACACAAA ATACTTCATC
2901  TTCGGCAACA AGAGAAACTA GAGGAGCTAG ATCCGAGAGC ACATCGCGAA
2951  ATAGAAGGAA TAGCAAATAA TCAGTCAAAT TAAAAATGTT AATTATAACA
3001  AAACCATTGA AACTAATGGA TAATAATGCA GTATGTGGAT TCCAAAAGAA
3051  AAAAGTCGAA GAATATAGGT GTAATTTATT TCTTAAAACA TTGTTTTGGA
3101  GTTTAAATTA ATAGTTAAAA AGTTAATGAG ATTAATTAAT CGTATCACAC
3151  TAGATGTATG TAATTTATTA GAGAATAAAA AAAAAAAAAA AA
```

Figure 4.9B. The predicted amino acid sequence of the NC1 gene.

```
  1  METPSPKAVP PPKPKRTKKP LAQTAPVGSE EKEDKKVEDT EPKPQEPITE
 51  TKPEPEPQQP QPITEPITEP QPKEPEPEEP KEQSMPESGP EGPKETEDKD
101  ATGPEEPKQT EPEHEKPKEQ DDTEPKPDTE HEHEKHDSQE DKDTKERQEQ
151  EDSDKKDDSV PEPEPEEPKD IDHVDVDEPE QEEQEDSDKK EDETEDKHEP
201  PKEQEDMEAG DTEQEPQPQP EGQEETEPEV SKDTEPKSEP IQDPIPDTKH
251  EDEQEQEDSD KEKPDAVPEE PKDTDHKQES EEDKDTKEPE EPTQPTEPEP
301  EPQPQQEVQP EEPTKPQPQE TKDPDDKQEE EKKPDPGEED RTDLDKDGQM
351  KTPEKMKPED IGGPEFPEPV DQSTPNTVKP PIFDKDRIKT PRLVFLTDDD
401  DGEGSEPTKR RKPFLIYDRP GKTPRRPKKK KIKQLFEHDD DGDGDDEDED
451  EDEESDEAEA EIHTSGTKDR KDDGGDKDDG SGGSGGAGPT PSTDENNQAG
501  GSGSGEAKHD DNGDSNTGGS KQSDTGNGDD SKDKDEKQQG EDEKEKEKEK
551  KEVEQKLPVT ETPSTPAISN PVTKTPKTPK KRRRPSKIPI KQTKVKPASR
601  PSTTPGKGPF KKPIVTPGGP SIVKPVSRPS TSRPSSTTPP TTTSGRQPPK
651  SDDRRSSQPP RPPFVVPWAT KEILTKGATK TPQQEQGTQF STEDRTREQG
701  KRAGGETRPD TSKGAVGEGR SRRSQRLSAR GTQNTSSSAT RETRGARSES
751  TSRNRRNSK*
```

Sequence homology searches with the NC1 sequence in the EMBL database showed that the NC1 sequence had low homology to the cell surface glycoprotein 1 precursor of *Clostridium thermocellum* (26.4% identity in a 732 amino acid overlap), the glutamic acid-rich protein procursor of *P. falciparum* (21.9% identity in a 565 amino acid overlap) and the sporozoite surface protein 2 precursor of *P. berghei yoelii* (23.0% identity in a 534 amino acid overlap). There is also some homology to the circumsporozoite of *Plasmodium spp.*

4.3.6.2. Nucleotide sequence determination of clone NC10

The cDNA clone NC10 had an insert of approximately 3.5 kb in size. ExoIII deletion series were generated to allow sequencing of both strands. For the reverse strand a 1 kb *Kpn*I and *Xho*I fragment was subcloned into pBluescript. ExoIII deletions of this subclone and the remaining 2.5 kb insert were then carried out as described in section 4.2.12.

Analysis of the sequence data demonstrated that the insert of clone NC10 is 3.4 kb in length and an ORF extends from the first base through to nucleotide 3100 where a TAA stop codon exists. The insert of the NC10 lacked a start codon and to obtain the full ORF it was decided to clone an upstream genomic DNA fragment. For this purpose, a 600 bp *Bam*HI / *Stu*I fragment derived from the 5' end of clone NC10 was used to probe a Southern blot. The *Bam*HI site was located within the polylinker. In order to increase the amount of parasite DNA isolated from macroschizont infected cells, *T. annulata* Ankara D7 macroschizont infected cells were incubated at 42°C for 5 days, a procedure which greatly increases the number of parasite nuclei per infected cell. The parasite enriched DNA was digested with restriction enzymes that cut near to the 5' end of the NC10 insert clone but not within its first 600 bp or very close to the *Stu*I site (*Apa*I, *Dra*I, *Eco*RV, *Stu*I, *Xba*I). Digests were separated on duplicate agarose gels. One gel was used for Southern blotting and probed with the 5' *Bam*HI / *Stu*I fragment. Hybridisation and washes were carried out at 65°C. Southern blot analysis identified a 2.5 kb fragment for both *Dra*I and *Eco*RV digestion, a 0.5 kb fragment and a 10 kb fragment for *Xba*I and *Stu*I digestion, respectively. A very faint and fairly large DNA fragment, about 14 kb, was identified for the *Apa*I digestion (data not shown). From the duplicate agarose gel, DNA

fragments of sizes identified for *Dra*I, *Eco*RV and *Stu*I were excised and purified using the "Gene Clean 2" kit. The purified DNA fragments were ligated into pGem 7zf (+) cut with *Sma*I. Following transformation, colony lifts were taken (see section 4.2.15.1) and probed with the 600 bp *Stu*I/*Bam*HI fragment. Two positive colonies were obtained; one from the *Dra*I and one from the *Eco*RV digest. Sequence analysis showed that both had an insert of about 2.6 kb.

Nested deletions were created with Exo III digestion for both strands of the insert derived by *Dra*I digestion. The genomic clone was 2.6 kb of which 600 bp were identical to the cDNA clone. The sequence of the genomic DNA revealed an ORF beginning at 442 bp. The combination of the genomic DNA sequence and the cDNA sequence generated a 5,400 bp sequence that contains a substantial ORF located between nucleotides 480 and 5,063 (Figure 4.10A) and it encodes a protein of 1,540 amino acids (Figure 4.10B).

The predicted NC10 protein sequence consists of interspersed tandem repeats of amino acids within the central region of the polypeptide. These repeats, underlined in Figure 4.10B, overlap in some instances. Tetrapeptide sequences similar to those observed in the QP protein /PIM (Baylis *et al.*, 1993; Toye *et al.*, 1995a) were also present. The sequence QPXP (X being F, Q or P) is found three times. The sequence QPXQ (X being G, P, Q, F, S, T, N, L) occurs 19 times; QQXQ (X being P, T, G, S, Q or N) occurs 12 times; and QGQX (X being F, P, G, Y or I) is found 11 times. There is also an eight-amino acid repeat, $^{Q}/_{T}GYY^{Q}/_{G}PX^{Q}/_{P}$ (X being S, P, G, T or N) which occurred eight times.

A search of the amino acid sequence databases with the predicted NC10 polypeptide revealed that the best similarity is to wheat glutenin (31.1% identity in a 679 amino acid overlap AC P08489), then to the calcium binding protein of *Dictyostelium discoideumi* (37.6% identity in a 388 amino acid overlap AC: P35085) and to human procollagen alpha 1 (V) chain precursor (24.8 % identity in a 706 overlap AC: P20908). There are also some small regions of homology to the circumsporozoite surface antigen of *Plasmodium knowlesi, P.berghei yoelii and P. simium.*

Figure 4.10A. The nucleotide sequence of the NC10 gene. The numbers represent the sequence position with reference to the most 5' base. ATG start and TAA stop codons are underlined. The 'R' in the nucleotide sequence of NC10 indicates a sequence ambiguity.

```
   1  GAATTCGGTA CCCCAAATTC ATCATTTAAT CTTGTGTAAA TTAGGATTAT
  51  TGTAATCAGT GTCAGTGAAT AAAATATAAC GTATAATGGT ATATAAATTG
 101  TTTTATTTGA ATATTAAGAT CATTTGATTG TATTTTATAA AATAATTCAC
 151  GAATGCGTTT TATCTAAAAC TGCATTCGGA AAAATCGACA AGATTTAGAT
 201  TAAATCAAAA GAAAAAATTC TGAAATAACA AATTGATTTA TTTTTATATA
 251  TAATGAAAAA GTTAGTTAAT CGAGGACAGA TTAAGTAATT AATTACACAC
 301  AATTGGCAGT ATTAAGTGATA TAAGGTCGAG AGTGGTTAAG AAAAGTTCGA
 351  GACTATTTCA GCATATTATA CAGAGGTTAT GTGATTCAAT AACGTAACAG
 401  AGCAAAATAC CCTCAAATTG TTTTTTATTG GTATATGAAG AAATGAAATC
 451  GTTAATAATT TATATTTACA TAATTAAAGT CTTAGTTTAT TGCGGAAGAT
 501  TTGCTATCTT TGATGTAAAG GGCATTTATA ACACAGTTCA ATATATATTG
 551  AGGAAAAAAT CCGACAACTC TCTCCTGTGC ACTATTCTTA ATGTTAAAAA
 601  GAATAATGAG TTGTATGGAG TAGTGAGCAA TGATGAAATA TTGTGGATTA
 651  AGCAATCAAA AAAGGAAGAG TGTCAAGATG TGTTGGTTAC AAAACATCAG
 701  GGGAAGGCTA AGCTGGCGCT GCTTCAGATC CAACACTTCA AGAGCTTTTA
 751  TACTGTAAAA AGATTAAGGT ACTTTTTAGG TATATGGGTA CCAGTAAGCA
 801  AAGAATTATA TAATAAAATA GATAAAATTA TATCTTGGAA GTTTGATTAT
 851  AAAGAATCTG AAATCAGTGT GGAATTAACA GAAGAATTTG ATACAGCTGG
 901  ACTTATGGTG AACAATTTCT TTATCAATGA AGAAAATAAA ACAAGGTTGT
 951  ATCCACGACC AGGGTTCAAA ATAACTTTAG TTAACTATAG AAATAATGAG
1001  TTATGGTCAA AAAATGATTA TCAAGAGGAG GCCATTCAAA TCGAAATTGT
1051  AAAAACAGAT TCAAAAATAC AACGTCTTTA CGTAAGATAC GAGACTTATT
1101  ACGGAACAGC AGATACTATT TATTATAAAT GTTATCCGGA ACTAAGAAGG
1151  GTGGATAGAA AAGAGTATTT CATGGATGAT GATGACGACG ATGACGATGG
1201  AGATGATGAT TATTTACCAG ATGGAATTGA TAGGTATAGA AGAATAGGAA
1251  CAAGAGACGT GGATTCAAGT CACGGAACTC AACCTGGTGG ATCAAGTCAA
1301  ACCGGAACTA CCGATCAAGG TACAGAGGAT CAAGATCAGA GAGCAAGTAG
1351  TCCTGTTTAT TATGGAGTAT CAGAGCTTGT TCCACTACCA CTTGGCACAA
1401  GGCCGGCCAC ACCCTTACCT ACCCCGTCAA AACTGGTGGC TCCAACTCCA
1451  ATTAAACATT TCCCTGAGGG TATTATTCGT TGTTTTCATG TCGAAACTTT
1501  TAGTGAAATT GGATTGGTCC CCCAAAAAAC TGCTAAACAA AGAGAAGCTG
1551  CTAAACAAGT AACTATTCGA GTAGGTGGTA CAGATGATTC TCAAGGACAA
1601  TCTGAGCCAA CTCATTATAT ACCACAACAT ACTGAGGCTG TGTCACATGG
1651  TACACATTAT ACCCCAGGTC AAGTTACAAG ACATCCTACT CAGCCAATTC
1701  TAAGAATGCC GCAGCAGACT GCTCCTAGGC CAACAGGGCG GCCAAGACAT
1751  GCTCCCCCAG TTCCACATGT TCCAACACAA CCATTTCCGA CTACTTTAAT
1801  ACAACCGCCG CCGCCAAGAC CATCCGGAAT TACACCTTAT AATATAATTC
1851  AGGGGATTGG GCAACAACGA TTTCCAACTT CCCAGACAGA CAATAGAGCA
1901  GAGCAAGGCT CTAGAACAGG ATCAACTACT TCAAGTTCAA CACCTACTAA
1951  CAAAGACAAT AGTAAAAAAA CAACTGATGA CAGCGATTCA GATGGCTCAG
2001  ATCTTTTTGA TATTCAAGAG ACTGATGTTG TTACAGAAGT TCCCGAAAGT
2051  GACACTCGTA TAGATGAATT TCTTAATATG ATAACAAAAA CAGTTACGAG
2101  TTCGACTACA AGTGTAACCA CCACATCCAG ACAAACGGGT CAAGCATCGA
2151  AGGCCATTGT TGCGATAACA TCAAGTACTC CACAACCAGG CACTCAATTT
2201  GGAGATGCTT CAGGGTTAAG TCTATTTTCA TCAGGTGAAC TCACTAGGGC
2251  AAGAGAATCT GGAGGATCAG GAGGAATGCA TGCCATTAGT GACTCCACAG
2301  TGAGTAGTAC GGTATCTTCG TGGGATTATT CAAATGCAAA TCCGTTTCAG
2351  GATTATATAG ATATAGGGCA AATCCCAGAG GTTGTAACAT CTGACTTTAG
2401  CCAGGAAGAA ATTGAACTGG CTTCTAGATC ATTAACAAGT TCAATTGAAA
2451  GTGGTAGAAC TGGGAGCAGT GGTGATCAAA CTCCAACTCC GGGTCAAACG
2501  TTTTCACCGG CCATTATGGG TGATGGCAGA ACCCCCAGAT ATCCTAGCGC
2551  CCCCAGTGTA AGGCCTCAAT TTGGAGTTGA AGGTGGTTTA AAGAACGTTA
2601  TTGTTAGACC AAGCTCTCTA ACTGGTACAC AAACGGTTAG GGCCCCAGTG
2651  GTTAGAGCCC CAGTGTTCAT GCCACGTGAT CCTGAAACTA GATCCGAGTA
2701  CGAGGRACCA CCACGTAAGA GACCAAATAC AGGACAAACG GTTCAACCAT
2751  TTCCACAACC TCAACCTCAA CCTGGGCAAT TTCAATATCA GCCTCCTCAA
2801  GCTCAACAAC CTCAAGGACA ATTTGGGCAG GGGCAATTTC AACCTTCACA
2851  AACTCAATTT TCCCAAGGTT ATTATCAACC AAGTCAGCAA ACTCAACAAA
2901  ATCTTGGGCA GTATGGGCCG AGTCAAGGTT TTCAACCTCA ACAAGGTACA
2951  GGTTATTATG GGCCTAATCA ACCTTTCCAA CCTCAACAAC AAGGACAATT
3001  TACTCAAGGA CAACCATTCC AAGGACAAGG TGATCAAGGT CAGGGTTATT
3051  TTCTATCTAT GTTACAAGAA CAAGATCCAT ATAACTCAGG CCAACAATCA
3101  CATCAAGGAC AATTTGGACA GGGTAATTAT CAAACTAATC AACCAACTCA
```

Figure 4.10A continued.

3151 ACCAAATCAG GGCCAATATT CTCAAGGTTA TTATGGACCC AATCAACCTT
3201 TGCAACCTCA GCAATCCCAA CCTGGGCAGG GTTATTATCA GCCTCCTCCT
3251 CCTCAACAAC AACAACCTCA AAATCAATAT CATCAGGGGT ACTATGGACC
3301 TAATCAGCCT CAACAAAATC AGGCTCAACA GGGACAAATT CCATATTCTC
3351 AGGGACAACC TACTCAACAA CCTCAAGGTT ATTATGGTCC TCCTCAACAA
3401 CAGCAAACTC AAGGTTACTA TCAACCTACT CAACAACAAC CAACTCAACC
3451 TCAATTTTCA CAGGGTTATT ATGGACCTAA TCAAAATCAA CCACCTCAAG
3501 GACAATTTGG ACATGGACAA CGTCAACAAC CTCAAACAGG ATATTCTCAA
3551 GGTTATTATC AACCTGGCCA AAATACAGGA ACACCAAATA CACAGACTCA
3601 GAATATTGGT TCAACTCAGC AACAAGGCAG ATATGGTGAT AATTCAAATG
3651 GAGAAGATAA TCAGAGTAGT GATGATGAAG ATGGTGATAA TTTTTTGGTA
3701 ACAGAACAAA CTGAGAAAAC AACTGGAGGA CCAGATACCG GCGCAGGTTC
3751 CACCCCTCCA GTTCAGCAAA GACCAGTTGG TTCCCAACGA CCTTTAGTTC
3801 GTGCGCCAAT TTACAGACCA CCAACAACTC CAGTAGGACC AATAAGCCAA
3851 CAACCACCAG TAAGCGGTGT TGGAGGGAAA AGGAGATTAC AAACCGCTCC
3901 GACCCATTAT ATTCCAGAAA ATGCACGTGG TGATCGGACA GGGTTAGGGC
3951 CTGAAAATTC AGGTAGAACT CGGCCAACCG CTCACGGTAA TAATTATGTT
4001 CCAGTATATA AACGACCGCG CCAATCTCAA GGTTTAGATT TTCCTGAAGA
4051 AGATCCTGAA TATGAAGATG ACAATGACGA CGCAGAATCT TTTATACTGA
4101 ATGTCAACTC CGATGTTGAA GAAACAGAAG ATACAGAGCG ATCTAGAGAT
4151 AAAACTGCAC AACCTAGGCC ACAAGTGAAT CCAGAACAAT CATCTGTTGT
4201 CAGAAGCATT CTTAGAAAAT CTTTACTGGG ACAAGCAAGA GGAACTCAAG
4251 GGGATTCCGC TTCAAAGACA ACTCCAGCAG GAACAAGACA TTCTGGTGGT
4301 CAAAGTGATG GAAGTCAAGC TAGTGGAGGG GGTTCAGGAG GTGCAGATTC
4351 TAGAAGACCA GATAGTTCTC AATCGTCACA GTTTGATGGA GGATATCGGT
4401 CTGCACTTCA ACATGTTGAT TCCAGAGACG TTAGAAATGA TGACGATCCA
4451 AATGGTGAGT TTAGGAAAGA AGGATTTCCA CTAGACGTAC TCGAGTTGGA
4501 TGACAGTACG AAACATTACA CCGTGTTGGT TGACAAGCTA CTGAAAGTTC
4551 CCGTGGTTAT TATAATTCCA GCAGATGAAT CGTTCGTTGA GGAGATAACT
4601 TTAGGAAGTT TAATGATATG GAGGAGCGAA ACTGTAAAGT GTGTTAGAGT
4651 AAGATTAGTA GTTTCAGAGG GTAACTTCAT TGCTATTGAA TTGACCTTGG
4701 TTACTCCAAG GAGGAAAAAG TTCCATTTGT TCTTTAGATA CCATGACGGT
4751 TATTACAAAG GTGTGGCAAG ACCAAGGTTT AACGAAGCTA TTTACGAACA
4801 GGCTACAAAA GTTCAAAACA TTAAAAGATT AAGTAAAAGG ATCGACAAGG
4851 AAATTATGGA GGAAGAAAAG GCTAAAAAAG ATGAAAAGGG AAACAAAAAA
4901 GCGTTCAGTA GATGGGAAAG TGCAACATAC AGGGAGTCAG ATGAAACTGA
4951 AGATGAAACT GAATATGAAA ACAGGAGGTG GGATATACAA GAAATAATAA
5001 CAAGAGAGAA TGGCAATCAC ACGAACATCA TCAGGGGACC AAAGAACATA
5051 ACCCAGAGGA GATAATTGAA TAAATAAAAT AAACAAAGAA ACAGATCAAA
5101 ACAAATATAA CAGTCTCACT CAATTAAAAC AAATTAAAAA ATTAAAAACA
5151 GTGGTTTG**TA A**TGGCATTAG TGGAAAATTT TCAATGTAAA TTAGAATTTT
5201 TTAAAAATCG AGTTTTTTTA CCAAAAAATT AGCTGGAGAT TTTTTGAAAA
5251 TAATAAAAAT GAATCAAGAG TATAAATTTA TACATCTAAA TGGTTCCAAA
5301 AATAATCACG ATTCCCCATC GGATTCTAAT CGCAATAAAA TCTGAAAGGC
5351 AGCACACATT TAAAATGTGT CAGCTCAACT AATTTCTGTA GACTATGAAA
5401 AAAAA

Figure 4.10B. The predicted amino acid sequence of the NC10 gene. Lined arrows show repeated tetrapeptide sequences, QPXP, QPXQ, QQXQ and QGQX. Straight lines show the repeat of eight amino acids. The 'X' in the amino acid sequence of NC10 indicates a sequence ambiguity.

```
   1  MKSLIIYIYI IKVLVYCGRF AIFDVKGIYN TVQYILRKKS DNSLLCTILN
  51  VKKNNELYGV VSNDEILWIK QSKKEECQDV LVTKHQGKAK LALLQIQHFK
 101  SFYTVKRLRY FLGIWVPVSK ELYNKIDKII SWKFDYKESE ISVELTEEFD
 151  TAGLMVNNFF INEENKTRLY PRPGFKITLV NYRNNELWSK NDYQEEAIQI
 201  EIVKTDSKIQ RLYVRYETYY GTADTIYYKC YPELRRVDRK EYFMDDDDDD
 251  DDGDDDYLPD GIDRYRRIGT RDVDSSHGTQ PGGSSQTGTT DQGTEDQDQR
 301  ASSPVYYGVS ELVPLPLGTR PATPLPTPSK LVAPTPIKHF PEGIIRCFHV
 351  ETFSEIGLVP QKTAKQREAA KQVTIRVGGT DDSQGQSEPT HYIPQHTEAV
 401  SHGTHYTPGQ VTRHPTQPIL RMPQQTAPRP TGRPRHAPPV PHVPTQPFPT
 451  TLIQPPPPRP SGITPYNIIQ GIGQQRFPTS QTDNRAEQGS RTGSTTSSST
 501  PTNKDNSKKT TDDSDSDGSD LFDIQETDVV TEVPESDTRI DEFLNMITKT
 551  VTSSTTSVTT TSRQTGQASK AIVAITSSTP QPGTQFGDAS GLSLFSSGEL
 601  TRARESGGSG GMHAISDSTV SSTVSSWDYS NANPFQDYID IGQIPEVVTS
 651  DFSQEEIELA SRSLTSSIES GRTGSSGDQT PTPGQTFSPA IMGDGRTPRY
 701  PSAPSVRPQF GVEGGLKNVI VRPSSLTGTQ TVRAPVVRAP VFMPRDPETR

 751  SEYEXPPRKR PNTGQTVQPF PQPQPQPGQF QYQPPQAQQP QGQFGQGQFQ

 801  PSQTQFSQGY YQPSQQTQQN LGQYGPSQGF QPQQGTGYYG PNQPFQPQQQ

 851  GQFTQGQPFQ GQGDQGQGYF LSMLQEQDPY NSGQQSHQGQ FGQGNYQTNQ

 901  PTQPNQGQYS QGYYGPNQPL QPQQSQPGQG YYQPPPPQQQ QPQNQYHQGY

 951  YGPNQPQQNQ AQQGQIPYSQ GQPTQQPQGY YGPPQQQQTQ GYYQPTQQQP

1001  TQPQFSQGYY GPNQNQPPQG QFGHGQRQQP QTGYSQGYYQ PGQNTGTPNT

1051  QTQNIGSTQQ QGRYGDNSNG EDNQSSDDED GDNFLVTEQT EKTTGGPDTG

1101  AGSTPPVQQR PVGSQRPLVR APIYRPPTTP VGPISQQPPV SGVGGKRRLQ
1151  TAPTHYIPEN ARGDRTGLGP ENSGRTRPTA HGNNYVPVYK RPRQSQGLDF
1201  PEEDPEYEDD NDDAESFILN VNSDVEETED TERSRDKTAQ PRPQVNPEQS
1251  SVVRSILRKS LLGQARGTQG DSASKTTPAG TRHSGGQSDG SQASGGGSGG
1301  ADSRRPDSSQ SSQFDGGYRS ALQHVDSRDV RNDDDPNGEF RKEGFPLDVL
1351  ELDDSTKHYT VLVDKLLKVP VVIIIPADES FVEEITLGSL MIWRSETVKC
1401  VRVRLVVSEG NFIAIELTLV TPRRKKFHLF FRYHDGYYKG VARPRFNEAI
1451  YEQATKVQNI KRLSKRIDKE IMEEEKAKKD EKGNKKAFSR WESATYRESD
1501  ETEDETEYEN RRWDIQEIIT RENGNHTNII RGPKNITQRR *
```

When the sequences of the clones NC1, NC10 and the QP protein were compared, low homologies to each other were observed. The predicted amino acid sequence of NC1 shared a 26.6 % identity (57.5% similarity) over 240 amino acids and 18.9 % identity (46.8% similarity) over 598 amino acids with those of the QP protein and the NC10 polypeptide, respectively. The nucleotide sequence of NC1 showed a 54% identity over 610 bp and 54% identity over 310 bp with the QP and NC10, genes respectively.

The predicted amino acid and nucleotide sequences of clone NC10 showed 29.9% identity (50.5% similarity) over 239 amino acids and 59.6% identity over 141 bp with the QP gene. The region of similarity covered the Q/P rich region of PIM.

4.3.7. Northern Blot Analysis

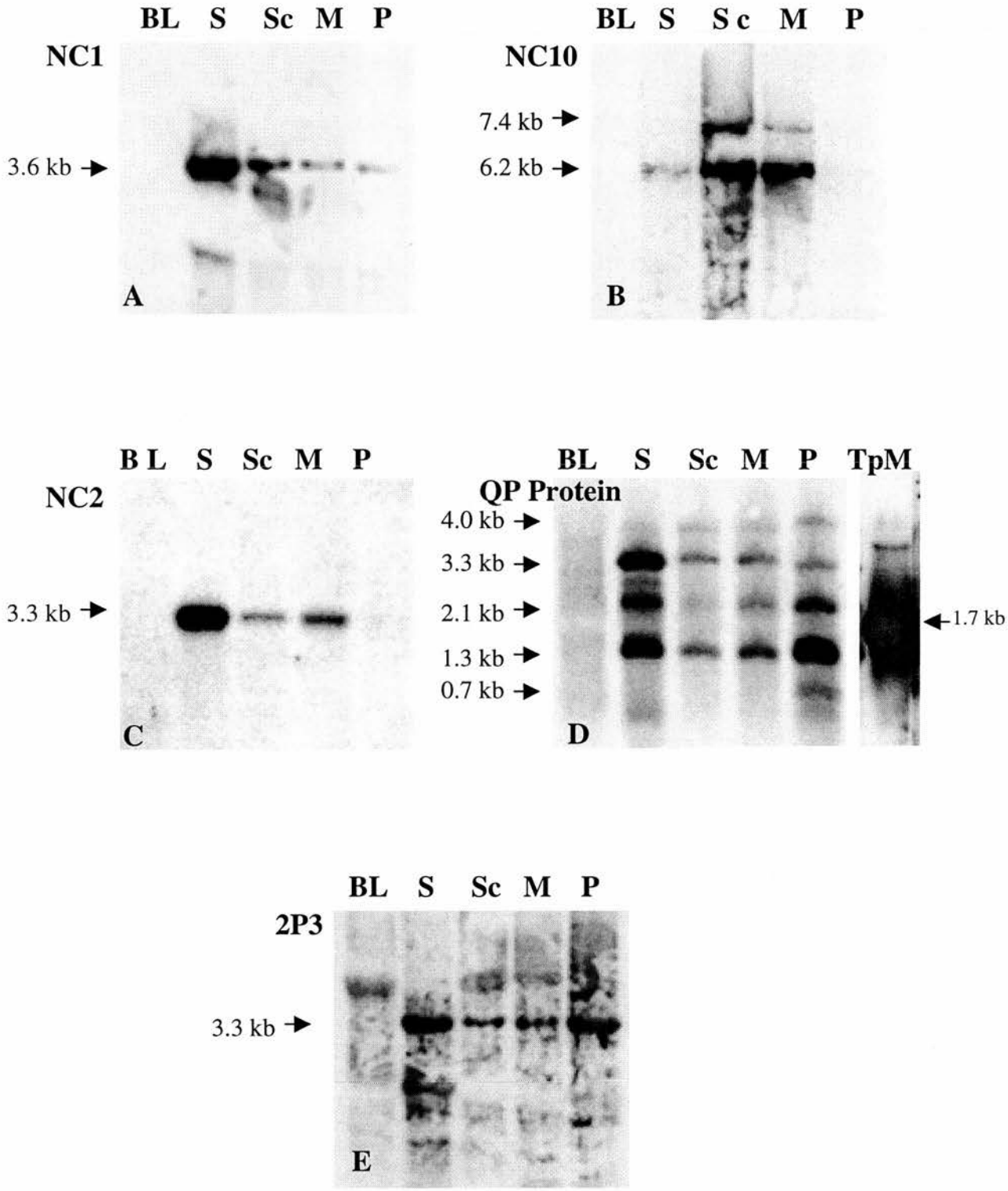
4.3.7.1. Expression level of cDNA clones throughout the life cycle stages

In order to study the expression of the clones isolated from the λ ZAP cDNA library and to show if indeed they are expressed by the macroschizont stage of the parasite, northern blot analyses were performed. Total RNA was isolated from different life cycle stages of the parasite; sporozoites, the macroschizont infected D7 cell line cultured at 37°C, the D7 cell line during differentiation towards the merozoites at 41°C and piroplasms (section 4.2.14.1). RNA from the bovine lymphoid cell line, BL20, was used as a negative control.

a) Group 1 (Clone NC1)

The mRNA profile of the gene represented by the cDNA clone NC1 is shown in Figure 4.11A. Although a single band of about 3.6 kb was detected in all life cycle stages, the results suggest that the gene represented by the NC1 clone was differentially expressed during the life cycle. The detected mRNA was found to be present at a high level in sporozoites and followed by a reduction upon differentiation to merozoites, and was detected at much lower levels in piroplasms and no signal was detected in BL20 RNA (Figure 4.11A). It can be concluded that transcription of the NC1 gene is downregulated following differentiation to the merozoite and is most likely expressed at high levels during sporozoite formation

Figure 4.11. Analysis of RNA levels during life cycle stages of *T. annulata* by northern blotting. Total RNA was isolated from the uninfected bovine cell line BL20 (BL), *T. annulata* Ankara sporozoites (S), clonal cell line D7 at 37°C (Sc), D7 cells under-going merogony at 41°C at day 6 (M), piroplasms (P) and *T. parva* Muguga macroschizonts (TpM). Ten μg of total RNA was loaded in each lane. (A) Hybridisation with the *T. annulata* NC1 cDNA probe, (B) hybridisation with the *T. annulata* NC10 cDNA probe, (C) Hybridisation with the *T. annulata* cDNA NC2 probe, (D) hybridisation with the QP cDNA probe and (E) hybridisation with the 2P3 probe. Northern blots of A, B, C and E were hybridised at 65°C and blot D was hybridised at 55°C. The sizes of mRNA species detected by cDNA probes are indicated in kilobase pairs (kb).


BL S Sc M P
NC1
3.6 kb
A
BL S Sc M P
NC10
7.4 kb
6.2 kb
B
B L S Sc M P
NC2
3.3 kb
C
BL S Sc M P TpM
QP Protein
4.0 kb
3.3 kb
2.1 kb
1.3 kb
0.7 kb
1.7 kb
D
BL S Sc M P
2P3
3.3 kb
E

and during the macroschizont stage. The size of the transcript is in slightly larger than the cDNA clone which was shown to be 3172 bp long by sequence analysis.

b) Group 2 (Clone NC10)

Figure 4.11B shows the results of the northern blot analysis using the clone NC10. This gene, like NC1, is differentially expressed during different life cycle stages. The gene represented by NC10 is expressed at high levels in the macroschizont stage and at low levels in the sporozoite stage of the parasite. No signals were detected in the RNA of the piroplasm stage of the parasite nor in the uninfected bovine BL20 cell line (Figure 4.11B). Two transcripts were visible in macroschizont RNA: the approximate sizes of the two mRNA species were around 6.2 kb and 7.4 kb. The 6.2 kb transcript was most prominent. Both mRNA species were larger than the size of the longest cDNA clone isolated. As indicated in section 4.3.6.2, sequence analysis of clone NC10 generated a 5400 bp sequence with an ORF beginning at 480 bp. This implies that there are about 800 bp missing to generate a 6.2 kb transcript and that there is about 1 kb of untranslated sequence before the ATG start codon. During the course of differentiation from macroschizonts to merozoites the messenger RNA species at 7.4 kb was down regulated while the mRNA species at 6.2 kb remained at approximately the same level. There was no signal detected by the NC10 cDNA probe with piroplasm RNA after an exposure period of two weeks. To confirm equal loading of total RNA, the same membrane was probed with a constitutively expressed gene, the large subunit ribosomal 2P3 probe (Swan *et al.*, 1996) (Figure 4.11E). As expected, although the 2P3 probe indicated that RNA levels from the macroschizont and macroschizont/merozoite stages were relatively less than those of the sporozoite and piroplasm stages, this did not effect the overall results with respect to the assessment of the relative expression pattern of the NC10 gene.

c) Group 3 (Clone NC2)

Figure 4.11C shows the result of the northern blot analysis with clone NC2. The expression pattern and the size of the signal were similar, but not identical, to that obtained for clone NC1. The mRNA detected by the NC2 probe was about 3.3

kb and was found to be differentially expressed throughout the parasite life cycle. Thus, the NC2 gene is highly expressed by the sporozoite stage and at a lower level by the macroschizont. The expression level stays possibly about the same during differentiation from macroschizonts to merozoites. The level of expression was very low but could be detected in the piroplasm stage.

d) QP protein

To determine the expression profile of the QP gene, RNA from different life cycle stages of *T. annulata* was hybridised with the QP cDNA probe at 55°C. RNA from *T. parva* Muguga macroschizonts was used as a positive control. Three major signals were detected in *T. annulata* RNA (Figure 4.11D) and the approximate sizes of these signals were 1.3 kb, 2.1 kb and 3.3 kb. There was also low level detection of a RNA species at 4.0 kb in the macroschizont, macroschizont/merozoite and piroplasm RNA samples and an additional 0.7 kb signal in piroplasm RNA. The expression level of these transcripts varied between life cycle stages. The expression profile and the size of the 3.3 kb fragment was very similar to the profile established for NC1 and NC2. Both the 2.1 and 1.3 kb signals were strongly expressed by both sporozoites and piroplasms. The expression level detected was low in the macroschizont and macroschizont/merozoite tracks. However, this could be due to lower amounts of RNA loaded for the macroschizont and macroschizont/merozoite stages than for the sporozoite and piroplasm stages (Figure 4.11E). The QP protein gene was found to be expressed in macroschizonts of *T. parva* at very high levels and encodes an mRNA species of 1.7 kb, as reported previously (Baylis *et al.*, 1993). In addition, a minor mRNA species of approximately 4.0 kb was also detected.

4.3.7.2. Different Parasite Stocks

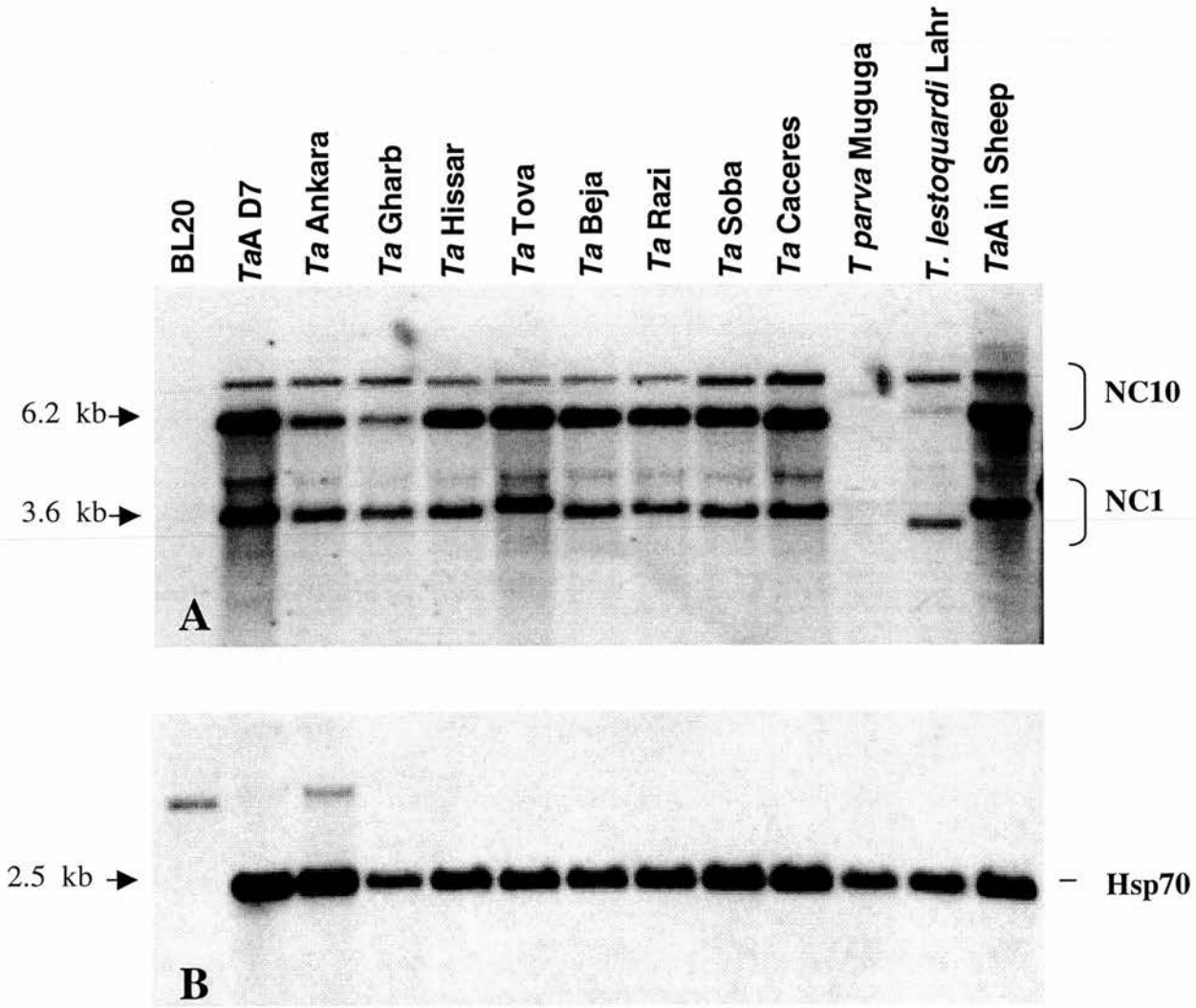
In order to determine the expression profile detected by the cDNA clones in RNA from different *Theileria* stocks, total RNA was isolated from eight geographically discrete *T. annulata* macroschizont infected cell lines (Table 4.1). Three of these stocks (Ankara, Hissar and Gharb) were previously shown to be different on the basis of isoenzyme analysis (Melrose *et al.*, 1980) and by their reactivity to a panel of anti-macroschizont mAbs (Shiels *et al.*, 1986). Additionally,

RNA was isolated from macroschizont infected cell lines representing *T. parva* Muguga and *T. lestoquardi* Lahr in order to examine the relatedness of the genes across other *Theileria* species. RNA from BL20 was used as negative RNA control and RNA from the D7 cell line was used as a positive control. As a further control for *T. lestoquardi,* RNA isolated from sheep lymphocytes infected with *T. annulata* macroschizonts was also included.

Figure 4.12 shows the results of the northern blot analysis using plasmid DNA from clones NC1 and NC10 as probes. All hybridisations were carried out at 65°C. Hybridisation using the NC1 and NC10 probe was carried out simultaneously to the same northern blot. The different stocks of *T. annulata* demonstrated a similar hybridisation pattern with NC1, with the exception of minor differences in size and expression levels in some RNA samples (Figure 4.12A). In order to confirm that the RNA was loaded equally, the blot, was stripped and re-hybridised with the heat shock protein 70 (Hsp70) probe (Mason *et al.*, 1989). The Hsp70 probe detected a 2.5 kb signal in the three *Theileria* species and all *T. annulata* stocks. An additional signal larger than 2.5 kb was detected in BL20 and *T. annulata* Ankara RNA. The results also showed that the amounts of RNA loaded was slightly different between stocks (Figure 4.12B) and coincided with the expression levels of cDNA clone NC1. An obvious size difference of the major detected mRNA transcript was observed only for the *T. annulata* Tova cell line. No hybridisation was detected in *T. parva* Muguga lane, whereas the probe clearly reacted with *T. lestoquardi* Lahr. The band intensity in *T. lestoquardi* Lahr is relatively lower than that observed for *T. annulata* which might be due to sequence differences between the genes of these two species. The size of the *T. lestoquardi* transcript is also slightly smaller. Considering that the hybridisation was carried out at high stringency conditions, *T. lestoquardi* is likely to possess good homology to the NC1 gene of *T. annulata.*

The NC10 gene was also shown to be expressed by all the *T. annulata* cell lines tested. In contrast to NC1, the size of the detected bands did not differ between the parasite stocks. The level of RNA loaded from *T. annulata* Gharb was lower than the other RNA samples and is likely to account for the reduced signal detected in this sample (Figure 4.12A). As with the NC1, there was no hybridisation signal detected in *T. parva* Muguga RNA. However, both bands at 6.2 kb and 7.4 kb were detected

Figure 4.12. Expression of NC1 and NC10 in geographically different *T. annulata* stocks and cross reactivity to *T. parva* and *T. lestoquardi* by northern blotting. The northern blot was prepared with 10μg of total RNA each from uninfected BL20 cells and macroschizont infected cells. (A) The blot was simultaneously probed with the NC1 cDNA probe and the NC10 cDNA probe. (B) The filter was then stripped and reprobed with the *T. annulata* (*Ta*) Hsp70 probe.


BL20
TaA D7
Ta Ankara
Ta Gharb
Ta Hissar
Ta Tova
Ta Beja
Ta Razi
Ta Soba
Ta Caceres
T parva Muguga
T. lestoquardi Lahr
TaA in Sheep
6.2 kb
3.6 kb
NC10
NC1
A
2.5 kb
Hsp70
B

in *T. lestoquardi* Lahr RNA. The intensity of the band at 6.2 kb was very low while the band at 7.4 kb was similar in intensity to that obtained with the other cell lines.

4.3.8. Protein Expression of cDNA Clones NC1 and NC10

Previous work indicated that the NC1 and NC10 genes are expressed at reasonable levels in the macroschizont stage of the parasite. To characterise the potential of these genes in the development of diagnostic ELISAs utilising recombinant antigens, it was necessary to obtain the corresponding recombinant proteins using prokaryotic expression systems.

4.3.8.1. Expression strategy

Different strategies were undertaken to express the cDNA clones NC1 and NC10 as recombinant polypeptides. The expression strategies will be explained for each clone separately.

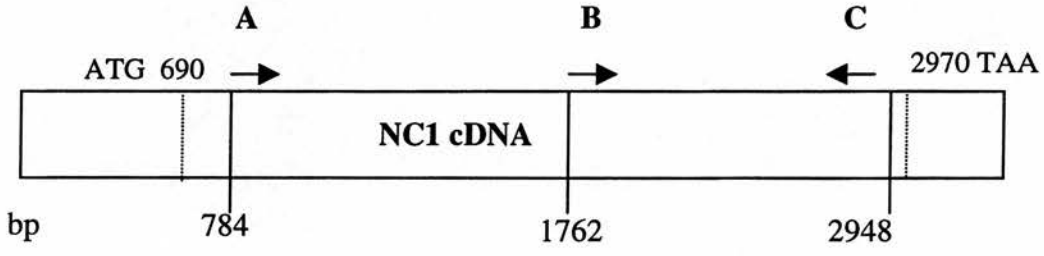
a) Clone NC1

The approach chosen to express NC1 was based on PCR amplification of parts of the gene and subcloning these, as no appropriate restriction sites were found in the gene sequence. The primers used are given in Table 4.4 along with the restriction sites incorporated into the primers for subcloning purposes. The fragments generated by PCR amplification are diagrammatically illustrated in Figure 4.13. The PCR primers A and C amplified 2.2 kb of the NC1 gene excluding the first 94 nucleotides and last 22 nucleotides of the coding sequence. The second primer combination used (B-C) amplifies 1,186 bp from the C terminus of the gene, starting at nucleotide 1762 and finishing at nucleotide 2948 of the cDNA clone. The pQE vector systems used to produce recombinant proteins are described in section 4.2.15.

Table 4.4. Primers used for creating a cDNA clone NC1 expression construct.

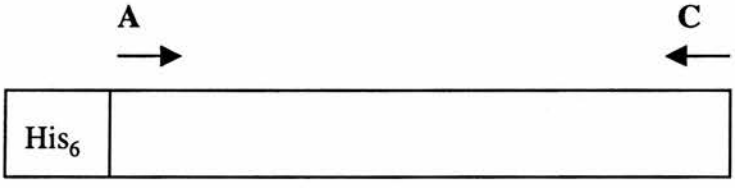
Primer	Sequence	Adaptor	Length
A	CCC GGG CTG ACC TGT TGG GAT CTG AGG	*Sma*I	27
B	GGA TCC CAG AGG ATA TTG GGG GAC CTG	*Bam*HI	27
C	GTC GAC GCG ATG TGC TCT CGG ATC TAG CTC C	*Sal*I	31

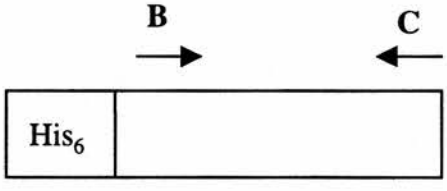
Figure 4.13. Construction of expression cassettes of the NC1 gene for the pQE vector systems. PCR amplification of NC1 DNA using primer combinations A/C and B/C resulted in DNA fragments of 2,164 bp and 1,186 bp respectively. These PCR products were ligated first into the pGem T Easy vector system and then subcloned into the pQE-31 vector digested with *Sma*I + *Sal*I or *Bam*HI + *Sal*I respectively. The resulting plasmids were trasnformed into *E. coli* M15[pREP$_4$].



1) ligation into pQE-31

2) transformation into M15[pREP$_4$]





Ten positive 2.2 kb-pQE-31 colonies were selected and analysed for protein expression. Upon induction of protein expression by adding of IPTG, cell growth was significantly reduced in comparison to the control cells containing the pQE-31 vector. Total cell extracts of uninduced and induced cells containing the 2.2 kb-pQE-31 construct were resolved on SDS-PAGE and visualized by Coomassie staining. A representative gel is shown in Figure 4.14. There was no evidence of an expressed fusion protein following induction. To increase the chance of expressing NC1, a smaller fragment (1.2 kb) was subcloned, avoiding internal start codons. The result of protein expression was very similar to that obtained for the 2.2 kb size fragment. The cell growth was hindered following IPTG induction and no obvious expression of a fusion protein was detected on SDS-PAGE (data not shown).

To overcome the lack of expression, two bacterial cultures containing the 1.2 kb- pQE-32 construct were grown until an OD_{600} of 2.4, rather than 1, was reached in 5 ml LB medium. The cells were then induced by adding IPTG to a final concentration of 4, rather than 2, mM. Total cell extracts were taken before and 2, 3 and 5 hours after induction and were separated by SDS-PAGE. No expressed protein was detected on the gels (Figure 4.15A). However, western blot analysis using anti-His-tag monoclonal antibody revealed that there was a very low level expression of a His-tag fusion protein at about 66 kDa (Figure 4.15B). In order to increase the expression level of the fusion protein two approaches were tested:

i) Cells were grown with a high concentration of ampicillin (200μg/ml), and supplemented with 2% glucose to reduce the level of fusion protein expression, as it might be toxic to the bacteria. Cells were grown until an OD_{600} of 0.9 was reached and were then induced. Samples were taken at the same time intervals as in (i).

ii) Cells were grown at a lower temperature, 30°C, and were then processed as above.

None of these methods led to an increase of fusion protein expression (data not shown). Although the level of protein expression was very low, recombinant fusion protein was purified from a large-scale culture using column chromatography as described in section 4.2.15. The amount of purified protein was low but levels increased with higher imidazole concentration in the elution buffer.

Figure 4.14. Coomassie stained SDS-PAGE gel of bacterial cell lysates containing the NC1 expression construct 2.2 kb–pQE-31. Total cell extracts of un-induced and induced bacterial cultures were separated by 10% SDS-PAGE gel. Lanes 1: un-induced cell extract; lanes 2,4-7: induced cell extract; lane 8: un-induced negative control; lane 9: induced positive control, which a part of *T. annulata* gene encoding the cdc2-related kinase and was kindly provided by Dr. Kinnard; lane 3: molecular size markers (kDa) as indicated on the left.

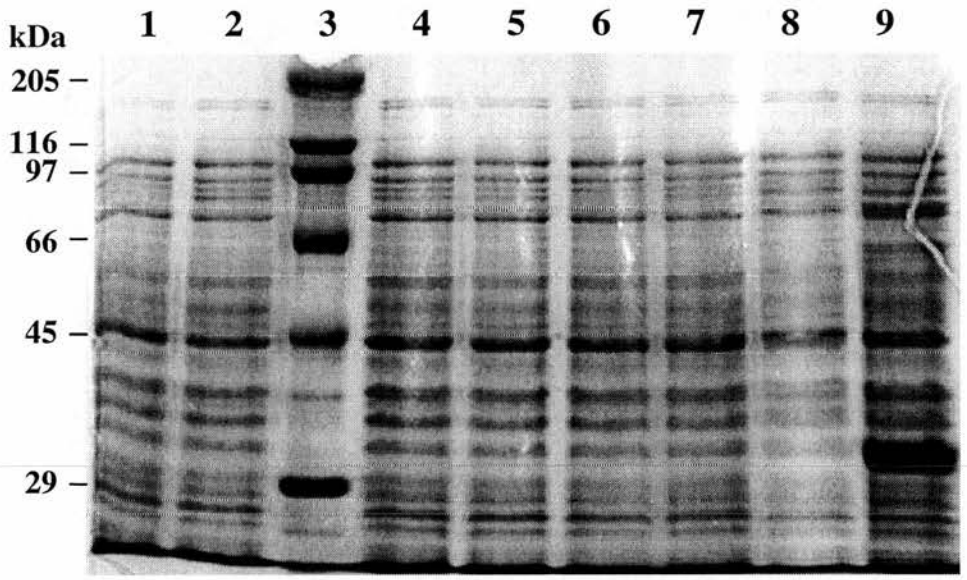
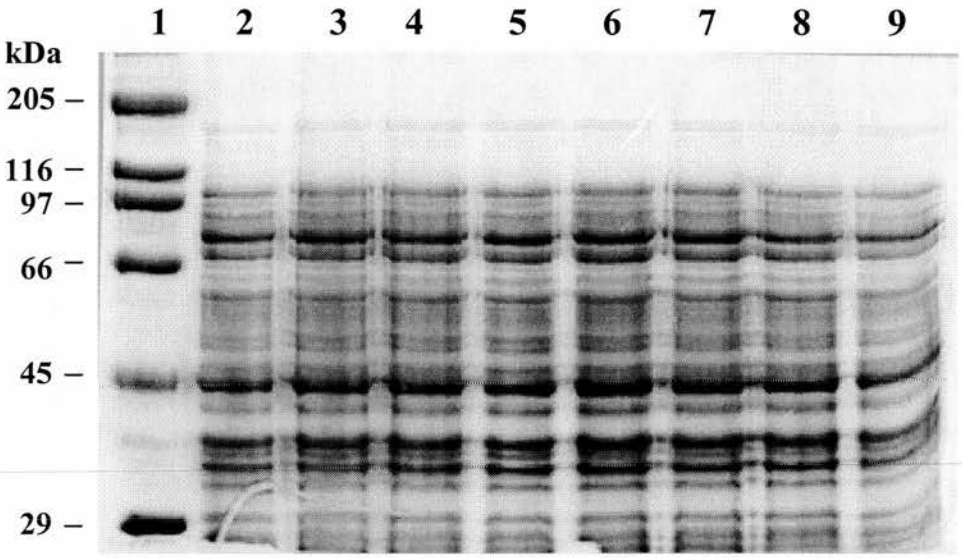

kDa
1
2
3
4
5
6
7
8
9
205
116
97
66
45
29

Figure 4.15. SDS-PAGE and western blot of protein extracts from bacteria expressing the NC1-1.2 Kb-pQE-32 construct using 4mM IPTG for induction. (A) Bacterial extracts were resolved on a 10% SDS-PAGE gel and visualised by Coomassie staining. (B) The resolved proteins were transferred onto a nitrocellulose membrane and probed with an anti-His-tag monoclonal antibody. Lane 1: molecular size markers (kDa) as indicated on the left; lanes 2 and 9: uninduced colony 1 and 2; lanes 3-5: colony 1 after 2 hours, 3 hours and 5 hours of induction; lanes 5-8 colony 2 after 2 hours, 3 hours and 5 hours of induction.

A.


1 2 3 4 5 6 7 8 9
kDa
205 –
116 –
97 –
66 –
45 –
29 –

B.

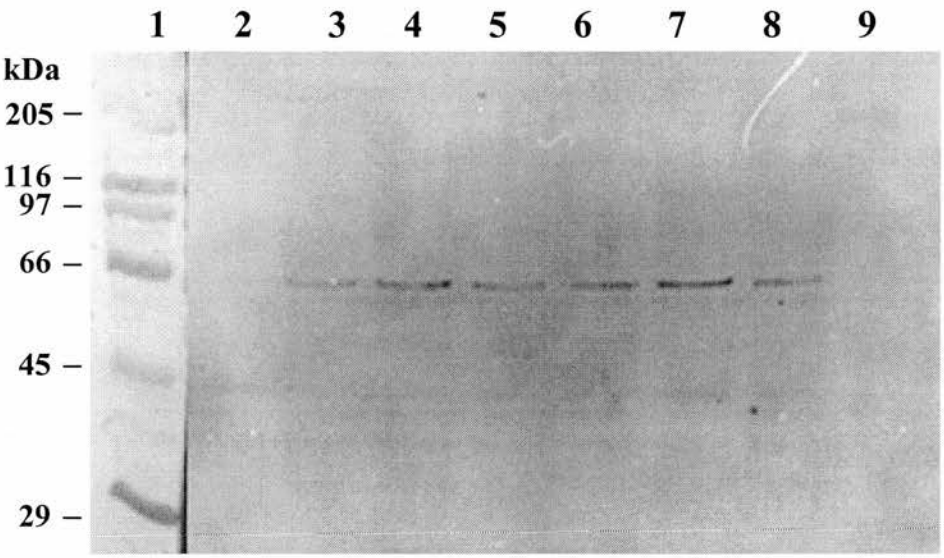

1 2 3 4 5 6 7 8 9
kDa
205 –
116 –
97 –
66 –
45 –
29 –

Figure 4.16 shows the His-tagged protein at 66 kDa molecular mass. A large number of non-specific bacterial proteins were co-purified from the column with the NC1 fusion-protein (Figure 4.16). A possible reason for the high concentration of bacterial protein contamination may have been due to the amount of Ni-Agarose used and as the amount of expressed fusion protein was low, excess Ni-Agarose can bind non-specifically to bacterial proteins.

To examine whether the purified protein was immunoreactive western blot analysis was carried out using three immune bovine sera. None of the immune sera reacted with the recombinant protein (data not shown) indicating that the protein isolated was not an immunodominant molecule.

b) Clone C10 (NC10)

The strategies undertaken to express a fragment of the NC10 gene as a recombinant protein are summarised in Figure 4.17. First, a 2.7 kb fragment of the NC10 cDNA was amplified by PCR using the primers listed in Table 4.5. A 2.7 kb amplification product was initially ligated into the pGEM T-Easy vector and then it was subcloned into the *Bam*HI/*Sal*I sites of both the pGex 5x-1 and the pQE-32 expression vectors (see section 4.2.15). Only a few positive transformed colonies were obtained with either plasmid construct. Sequence analysis confirmed that the ORF was maintained for both constructs.

Table 4.5. Primers used for creating a cDNA clone NC10 expression construct.

Primer	Sequence	Adaptor	Length
A	GGA TCC CGG GTC AAG CAT CGA AGG CC	*Bam*HI	26
B	GTC GAC GCT TCG TTA AAC CTT GGT CTT GCC ACA CC	*Sal*I	35

Following growth and induction of positive colonies, it was evident that the expression levels were so low that they could not be detected on Coomassie stained SDS-PAGE gels. However, western blot analysis of the total cell extracts determined low level expression, from both systems, of immunoreactive bands at around 100 kDa, which were detected by immune bovine sera (data not shown). Attempts were then made to purify these proteins and while this was successful for the GST-fusion

Figure 4.16. Purification of the recombinant pQE-NC1-1.2 protein. (A) 1 ml fractions were collected from the column and 20 µl of each fraction were resolved on a 10% SDS-PAGE gel and visualised by Coomassie staining. (B) The resolved proteins were transferred onto a nitrocellulose membrane and probed with an anti-his-tag monoclonal antibody. Lane 1: molecular size markers (kDa) as indicated on the left; lanes 2-5, 1 ml fractions of protein eluted using 0.3 M imidazole; lanes 6-9, 1 ml fractions of protein eluted using 0.5 M imidazole.

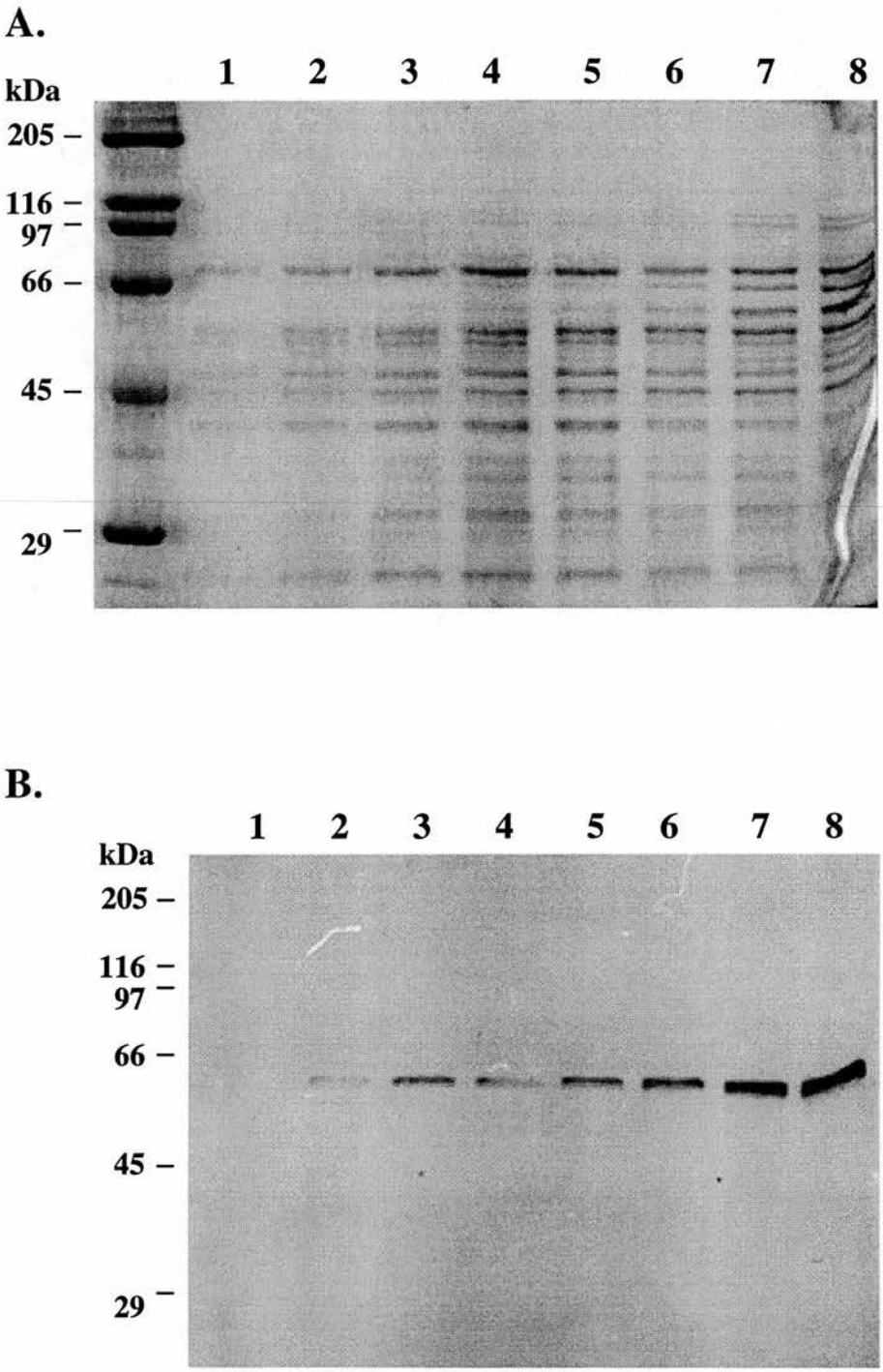
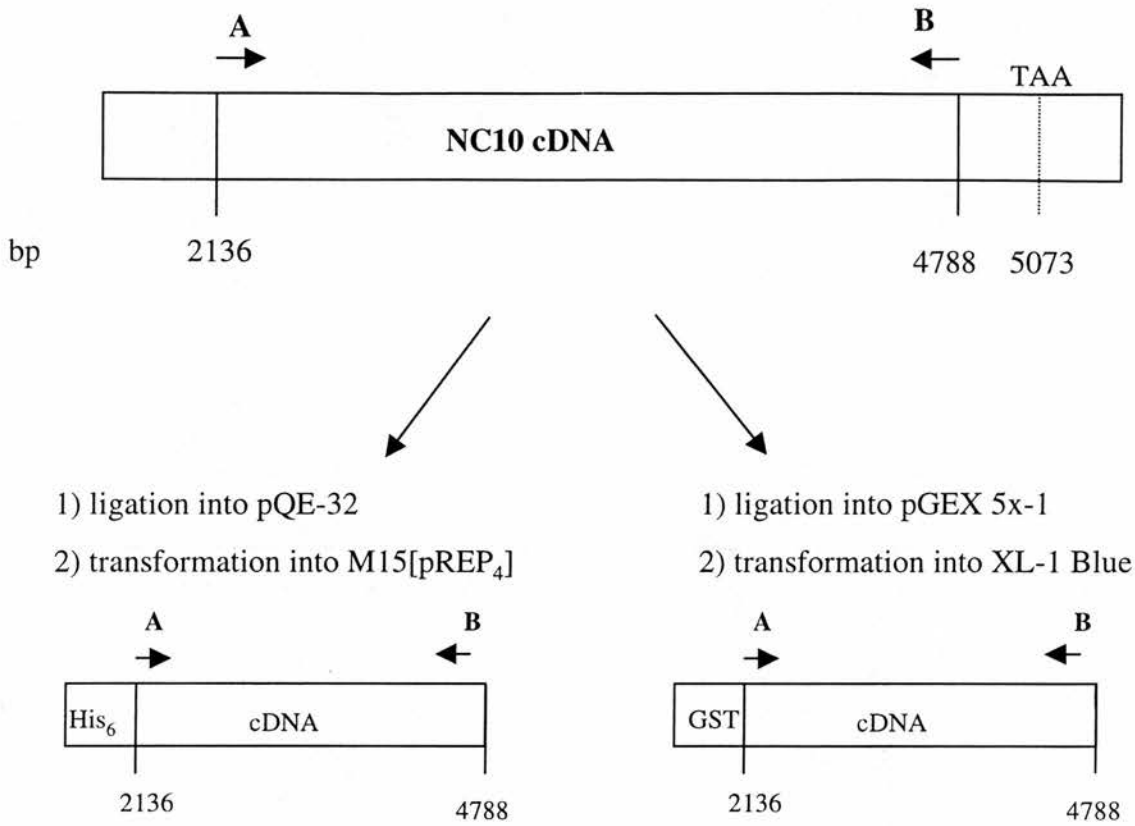

A.
1 2 3 4 5 6 7 8
kDa
205
116
97
66
45
29
B.
1 2 3 4 5 6 7 8
kDa
205
116
97
66
45
29

Figure 4.17. Construction of expression cassettes of the NC10 gene for the pQE and the pGex vector systems.

Design 1: PCR of the NC10 gene using primers A and B resulted in a 2,652 bp product. The PCR product was first ligated into the pGem T Easy vector, and then subcloned into the pQE-32 and pGex 5x-1 digested with *Bam*HI + *Sal*I. The resulting plasmids were used to transform *E. coli* M15[pREP$_4$] and XL1 blue, respectively.

Design 2: The fragment obtained in design 1 was digested with *Stu*I + *Xho*I which resulted in a 2 kb fragment which was ligated into pQE-32 and pGex 5x-1 digested with *Sma*I + *Sal*I. The resulting plasmids were used to transform *E. coli* M15[pREP$_4$] and XL1 blue, respectively.

Design 1



Design 2

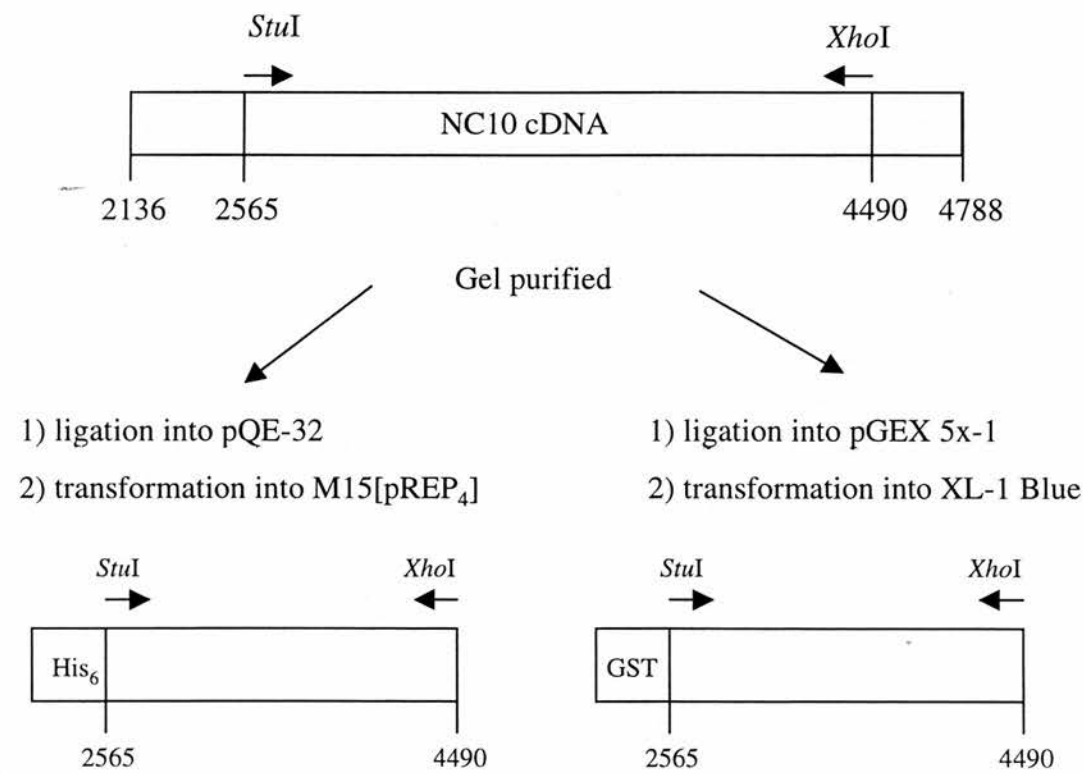
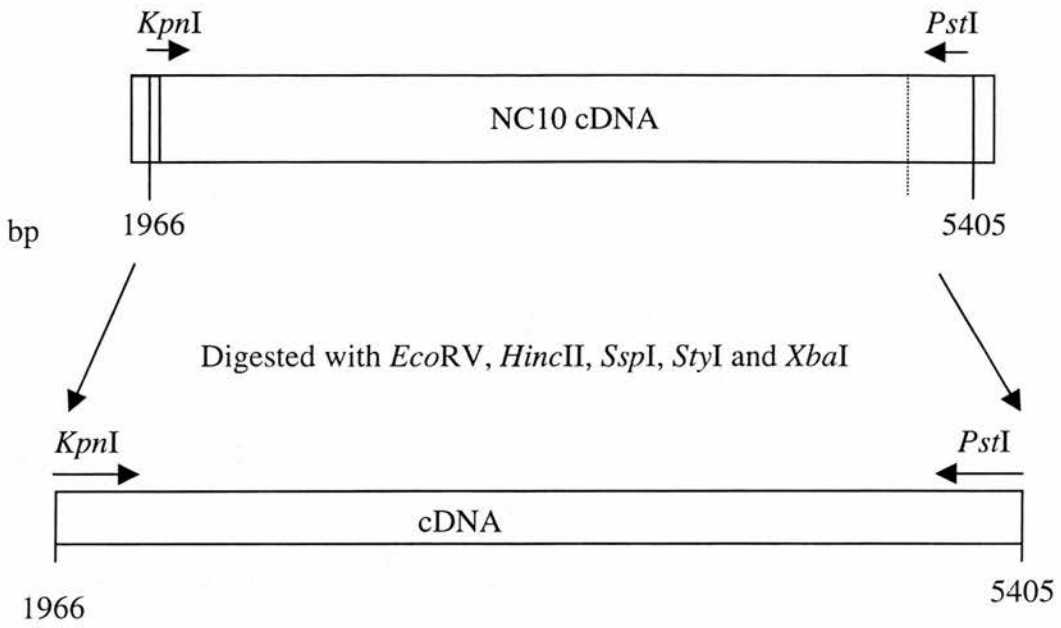
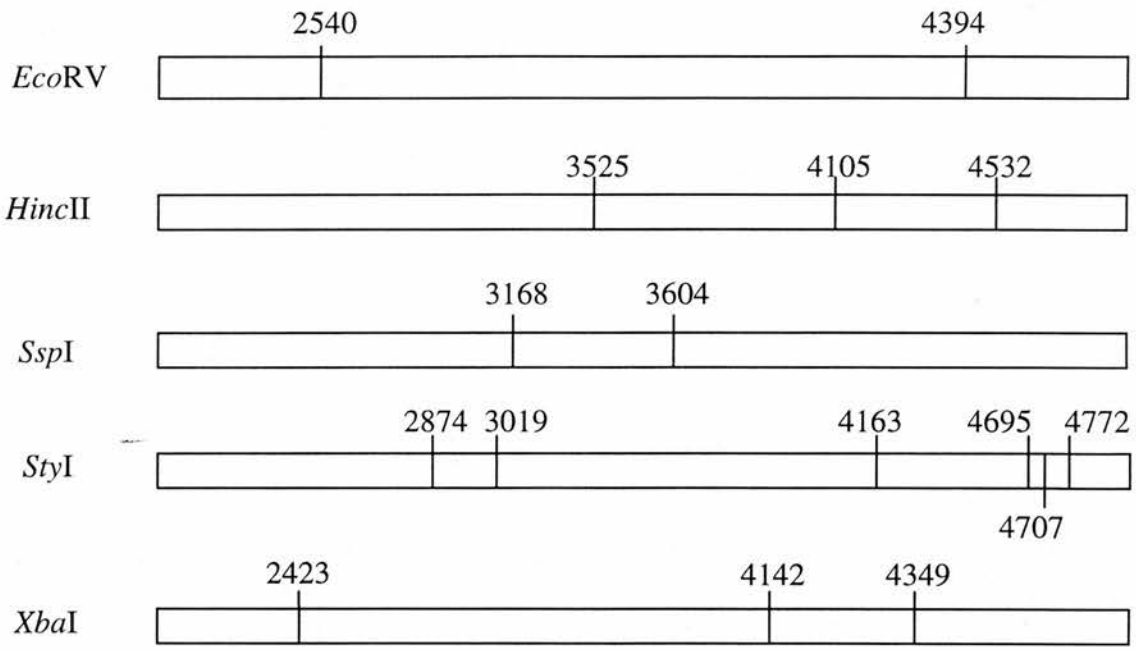


Figure 4.17. continued

Design 3: The *Kpn*I-*Pst*I fragment of the NC10 cDNA was isolated by gel extraction and then digested with *Eco*RV, *Hinc*II, *Ssp*I, *Sty*I and *Xba*I. The resulting DNA fragments were blunt ended and ligated into the pGEX 5x-1 and pGex 5x-2 digested with *Sma*I. The resulting plasmids were used to transform *E. coli* XL1 blue.

Design 3





ligated into pGEX 5x-1 and pGex 5x-2

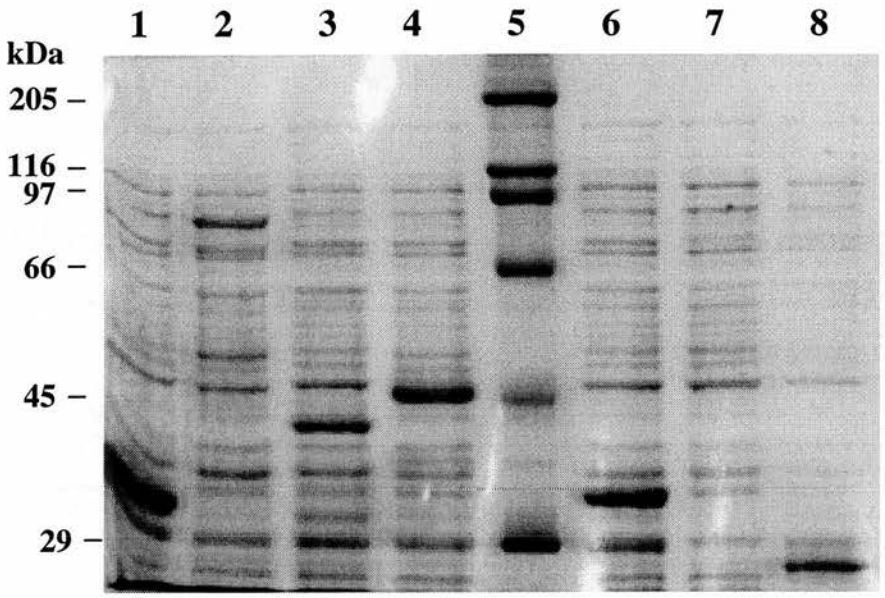
protein, the His-tag protein could not be purified following standard methodology. SDS-PAGE analysis of the purified GST-NC10 fusion protein revealed a band of around 100 kDa and many smaller products which all reacted with immune bovine sera, indicating that the polypeptide encoded by NC10 is immunogenic (data not shown).

Since expression of the large fragment of the protein resulted in very low yields, it was decided to sub-clone and express smaller immunogenic fragments of NC10. The NC10 cDNA clone was digested with *Stu*I / *Xho*I releasing a 2 kb fragment spanning the QP repeat region of the gene which was ligated into the pGEX 5x-1 and pQE-32 vectors. Protein expression levels of these constructs were examined using 10 positive colonies for each system. Although sequence analysis confirmed that the ORF for the fragment was maintained, there was no visible expression on SDS-PAGE gels. Several approaches described in section 4.3.9.1-(a) failed to improve the levels of protein expression.

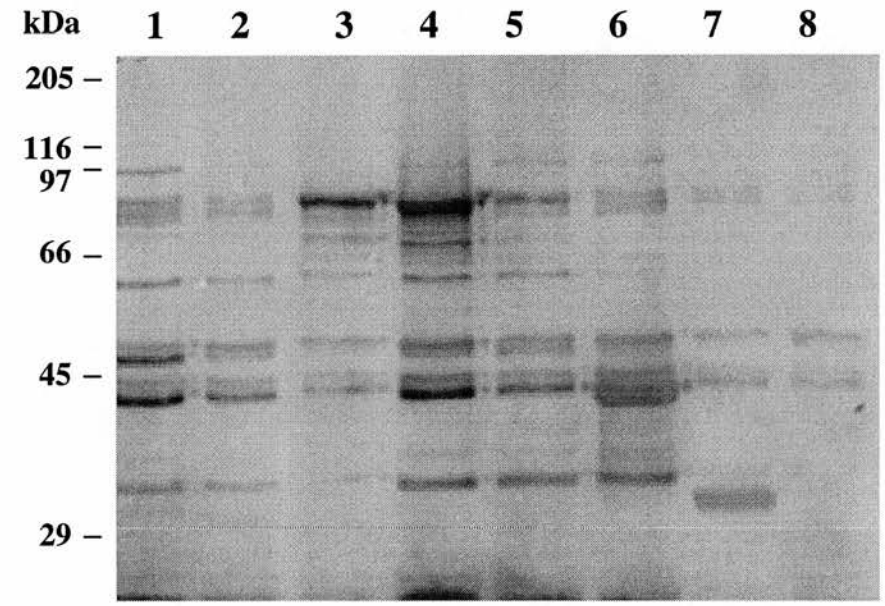
Another methodology used to generate an NC10 expression clone was based on isolating the *Kpn*I-*Pst*I fragment of the NC10 cDNA clone followed by restriction digestion with *Eco*RV, *Hinc*II, *Ssp*I, *Sty*I and *Xba*I (Figure 5.17 Design 3). These enzymes recognise multiple sites in the sequence of the gene. The resulting DNA fragments were blunt ended and cloned into pGex 5x-1 and pGex 5x-2. Colony lifts were taken and probed with the *Kpn*I/*Pst*I fragment (see section 4.2.15). Positive clones were tested their ability to express recombinant NC10 protein. 8 colonies derived from the pGex 5x-1 *Eco*RV ligation, 1 from the pGex 5x-1 *Hinc*II ligation, 6 from the pGex 5x-1 *Ssp*I ligation, 3 from the pGex 5x-2 *Eco*RV ligation, 1 from the pGex 5x-2 *Hinc*II ligation, 7 from the pGex 5x-2 *Ssp*I ligation and 1 from the pGex 5x-2 *Sty*I ligation expressed proteins which were visible by Coomassie staining. Figure 5.18A shows seven of these proteins on a Coomassie stained SDS-PAGE gel. Western blot analysis, however, demonstrated that only two of these recombinant proteins were specifically detected by immune bovine sera: *Ssp*I (13) and *Ssp*I (24) (Figure 4.18B). Because of time constraints work was concentrated on the pGex 5x1-NC10 - *Ssp*I (13) construct, since it produced a larger protein and reacted more intensely with immune bovine serum.

Figure 4.18. Expression of NC10 gene fragments following digestion with several restriction enzymes. (A) Coomassie stained 10% SDS-PAGE gel. Lanes 1-4 and 6: total lysates of induced bacterial cultures containing expression constructs: pGex-5x1-NC10-Ssp4, pGex-5x1-NC10-Ssp13, pGex-5x2-NC10-Ssp16, pGex-5x2-NC10-Ssp24 and pGex-5x2-NC10-Sty40 fragments; lane 7 and 8: uninduced and induced pGex 5x-1 controls, lane 5: The molecular weight marker (kDa) as indicated on the left side of the gel. **(B)** Western blot analysis of the expressed NC10 fragment. Lanes 1-2: the pGex-5x1-NC10-Ssp4 lysates before and after induction; lanes 3-4: the purified pGex-5x1-NC10-Ssp13 protein and induced whole cell lysates; lane 5: induced pGex-5x2-NC10-Ssp16 lysate; lane 6: induced pGex-5x2-NC10-Ssp24 lysate; lane 7: induced pGex-5x2-NC10-Sty40 lysate; lane 8: induced pGex-5x1 lysate for control of GST fusion protein. The size of the molecular weight markers is shown on the left of the gel in kDa.

A.


1 2 3 4 5 6 7 8
kDa
205 –
116 –
97 –
66 –
45 –
29 –

B.


kDa
1 2 3 4 5 6 7 8
205 –
116 –
97 –
66 –
45 –
29 –

To reduce possible non-specific immune responses against the GST-fusion protein fragment generated by GST, a 1.2 kb *Bam*HI - *Sal*I fragment (NC10-Ssp13) was ligated into the pQE vector system. The NC10-Ssp13 DNA fragment was purified from an agarose gel, ligated into a *Bam*HI-*Sal*I cut pQE-32 vector and transformed into M15[pREP$_4$] cells (construct called pQE-NC10-Ssp13). Six positive colonies were selected and analyzed for fusion protein expression. All six colonies produced a fusion protein of approximately 60 kDa, which was larger than the expected size of 45 kDa (Figure 4.19). The sequence of the cloning junctions indicated that the His-tag was in frame with the ORF of the NC10 gene fragment. The sequence analysis also revealed that the expression fragment was located between 1,966 bp and 3,168 bp of NC10 consensus sequence and contained the first half of the repeat region of the gene.

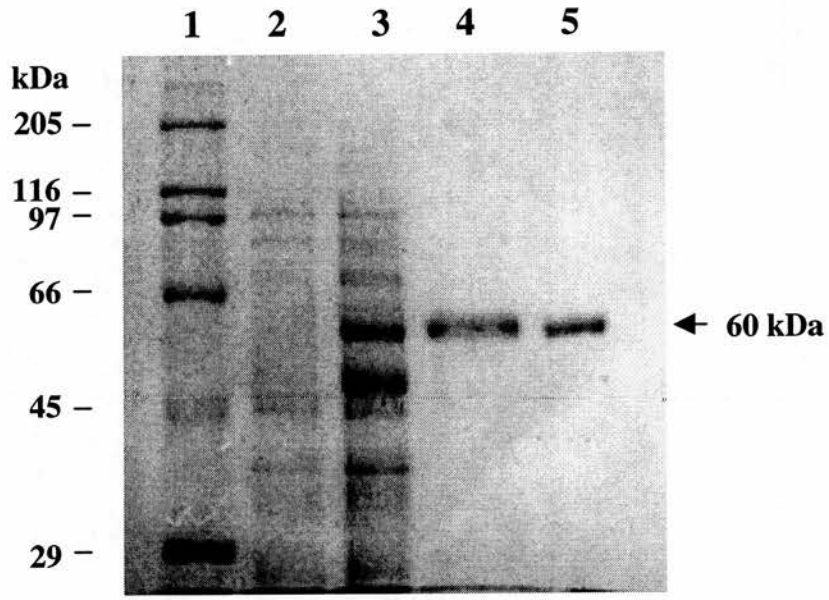
Before large-scale production of the fusion protein was performed, the solubility of the protein was examined as described in section 4.2.15.1. The result revealed that the protein expressed in M15[pREP$_4$] cells was not soluble.

An attempt was made to solubilize the protein first by using an EGTA solution, then by using lysozyme (section 4.2.15.1). Both techniques failed to solubilize the recombinant protein. Although low levels of solubilized protein was seen in the supernatant after lysozyme treatment, the amount was too low to justify this approach. Therefore, protein purification was carried out under denaturing conditions as described in section 4.2.15.1. Purification under denaturing conditions with Ni-NTA resin allowed the purification of a single polypeptide migrating at approximately 60 kDa as determined by SDS–PAGE (Figure 4.19). The protein was refolded by gradually reducing the concentration of urea. The protein remained soluble in 50 mM Tris.HCl pH 8.0 but came out of solution in PBS 7.4 and in 50 mM Tris-HCl pH 7.4.

4.3.9. Western Blot Analysis Using Antisera Raised Against the Recombinant His-tagged NC10-Ssp13 Protein

To characterise antigens that are recognized by an anti-NC10-Ssp13 rabbit serum, western blotting was conducted using protein extracts from macroschizont infected cell lines: eight geographically different *T. annulata* stocks, the

Figure 4.19. Coomassie stained SDS-PAGE gel showing the expression, purification and refolding of recombinant NC10-Ssp13 protein. Protein samples were resolved on a 10% SDS-PAGE and visualised by Coomassie staining. Lanes 2 and 3: uninduced and induced bacterial lysate containing the pQE-NC10-Ssp13 construct; lane 4: purified protein using the denaturation method; lane 5: recombinant protein following refolding by gradually reducing the urea concentration. The size of the molecular weight markers (lane 1) is shown on the left of the gel in kDa.


1
2
3
4
5
kDa
205 –
116 –
97 –
66 –
45 –
29 –
60 kDa

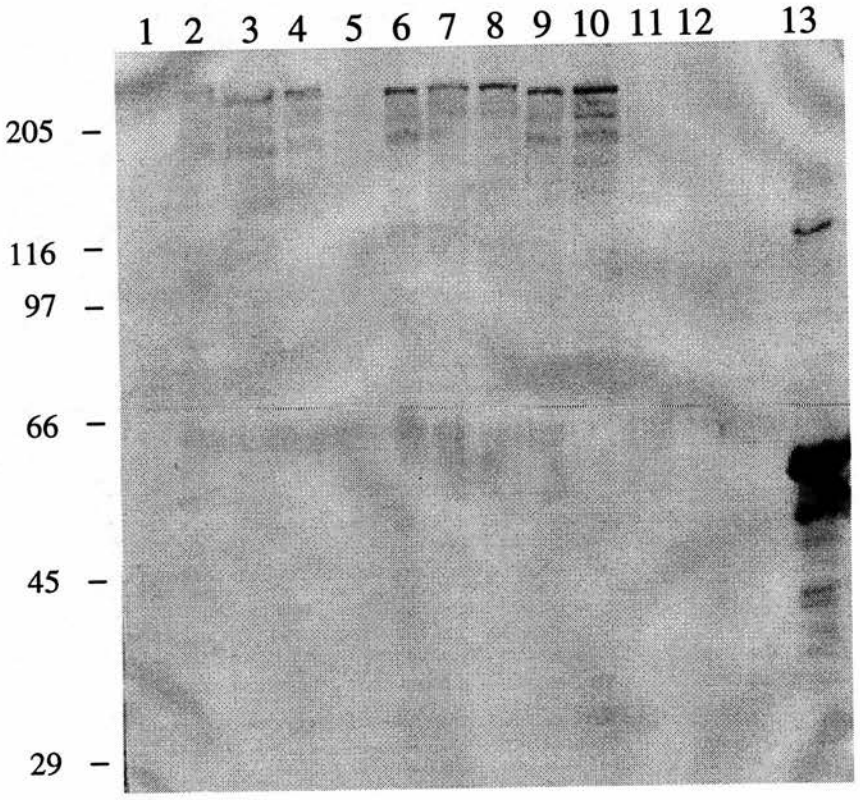
T. lestoquardi Lahr and the *T. parva* Muguga infected cell line (Table 4.1). Figure 4.20 shows the result of an immunoblot of total cell extracts from *Theileria* infected cell lines. All *T. annulata* cell lines reacted with the immune rabbit serum, except *T. annulata* Caceres from Spain. The experiment with the Caceres cell line was repeated two more times and identical results were obtained. The antiserum detected 3-6 bands in seven *T. annulata* stocks, whose molecular weight ranged between 150 kDa to above 205 kDa. The polypeptide just above 205 kDa was the most prominent polypeptide recognised by the serum and other bands were less intense. They may represent processed forms of the NC10 protein or a group of related proteins, which share the same/similar epitopes. Alternatively, the lower bands may be the result of proteolytic degradation during the preparation of the samples. It is unclear why the Caceres cell line did not react with the immune serum. It could be due protein degradation during lysate preparation or it might be due to antigenic variations.

The immune rabbit serum did not react with any polypeptides from *T. parva* Muguga cell extract. However, cross reactivity with *T. lestoquardi* was observed (Figure 4.20). The polypeptide detected in *T. lestoquardi* was slightly bigger than the prominent band of *T. annulata* but the bands detected were very faint (It is not possible to see them in the Figure 4.20). The immune serum did not react with BL20 (Figure 4.20) confirming specific recognition of a parasite polypeptide.

4.3.10. IFAT

The location of the NC10 protein within the macroschizont-infected cell was identified by IFAT using antisera raised against the His-tagged NC10-Ssp13 fusion protein in two rabbits. For this purpose, macroschizont infected cells of three stocks of *T. annulata* (Ankara, Gharb and Hissar) stocks and two *T. annulata* Ankara clonal cell lines (D7 and D7B12) were used. An uninfected lymphosarcoma cell line BL20 and the same lymphosarcoma cell line infected with *T. annulata* Ankara were used as negative and positive controls, respectively.

Figure 4.20. Western blot analysis of *Theileria* infected cell lines using rabbit serum raised against NC10-Ssp13 recombinant protein. Lane 1: uninfected BL20 cell line; lane 2: *T. annulata* Ankara infected BL20 cell line; lanes 3-10: *T. annulata* Ankara D7, Beja, Caceres, Gharb, Hissar, Razi, Soba, Tova cell lines; lane 11: *T. parva* Muguga, lane 12: *T. lestoquardi* Lahr, lane 13: recombinant protein NC10-Ssp13.

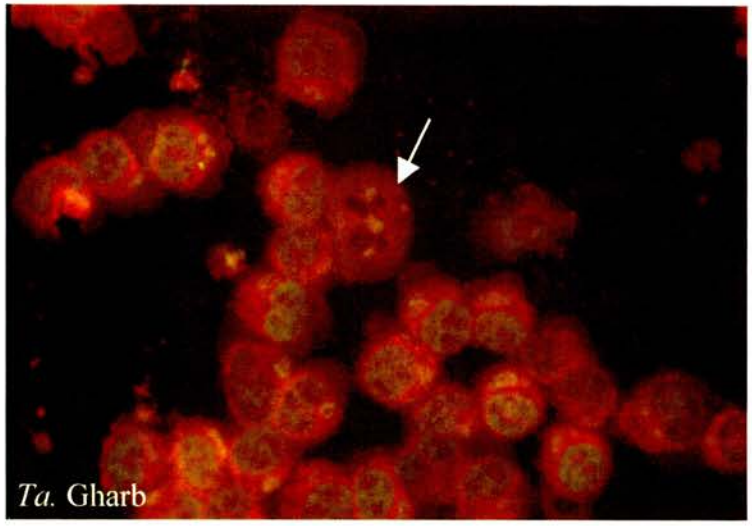

1 2 3 4 5 6 7 8 9 10 11 12 13
205
116
97
66
45
29

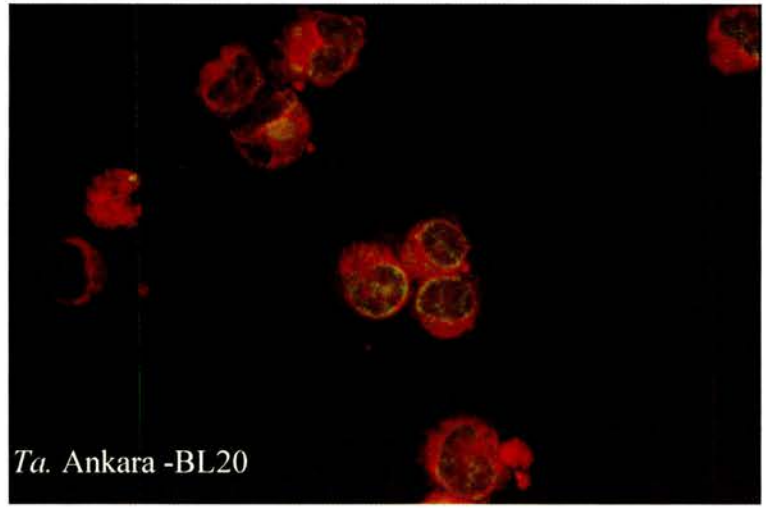
IFAT revealed that all *T. annulata* infected cells tested in the current study were recognised by both of the rabbit sera. Interestingly, the sera reacted with the nucleus of the infected host cell as well as the macroschizont within the cell (Figure 4.21). Immunofluorescence appeared to be distributed over the area of nuclear envelope but was excluded from the nucleoli (Figure 4.21). Although the general pattern of the reactivity was the same for all *T. annulata* cell lines, there were differences in the level of staining of the host nucleus between cell lines (Figure 4.21). Thus, the strongest reaction was obtained with the D7B12 cell line, while the weakest reaction was detected with the Gharb cell line. Variability in the level of staining was also observed within the cell lines. The host nuclei of some of the infected cells stained very strongly, whereas in other cells of the same cell line less intense staining was observed. Furthermore, the reactivity against the host nucleus was not observed in cells with condensed chromosomes, although the macroschizonts in these cells showed immunoreactivity and general staining could be observed throughout the host cytoplasm (see Figure 4.21). Recognition of the host cell, therefore, may be confined to cells that are in the interphase of the cell cycle.

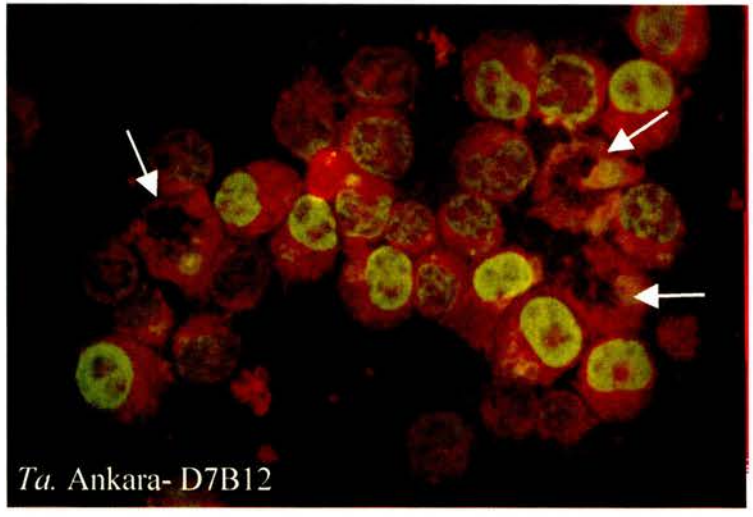
No staining of either macroschizonts or the host nucleus was observed with the pre-immune rabbit serum (data not shown). In addition, the antisera did not react with the uninfected BL20 cells. Results demonstrating that the nuclei of *T. annulata* Ankara infected BL20 cells, but not the nuclei of uninfected BL20 cells, reacted with the antisera suggest that the expression of the detected antigen is related to infection of the host cells by the parasite.

In order to determine if the pattern of reactivity obtained with anti-NC10-Ssp13 sera was species specific, IFAT was performed with macroschizont-infected cells of *T. parva* Muguga and *T. lestaquardi* Lahr. The NC10-Ssp13 antisera strongly reacted with macroschizonts of *T. parva* Muguga. However, no reactivity was detected against the nuclei of the host cells (Figure 4.21). In contrast, both macroschizonts and host nuclei of the *T. lestoquardi* Lahr infected sheep cells were stained with the antisera, although the staining was faint in both macroschizonts and nuclei (Figure 4.21). It should be noted, however, that the nuclei of occassional cells were strongly stained in the *T. lestoquardi* Lahr cell line.

Figure 4.21. IFAT analysis of macroschizont infected cell lines using the antiserum raised against NC10-Ssp13 recombinant protein. *T. annulata* Ankara infected BL20, *T. annulata* Ankara D7B12, *T. annulata* Hissar, *T. annulata* Gharb, *T. parva* Muguga and *T. lestoquardi* Lahr cell lines were tested. The uninfected BL20 cell line was used as negative control. *Ta.* denotes *T. annulata*; *Tp.* denotes *T. parva*; *Tl.* denotes *T. lestoquardi.* Arrows shows cells that are undergoing mitotic cell division.


Ta. Gharb


Ta. Ankara -BL20


Ta. Ankara- D7B12

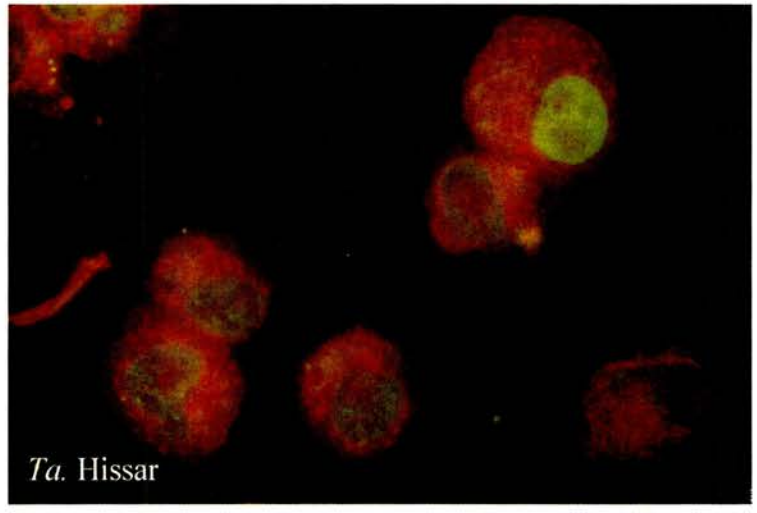
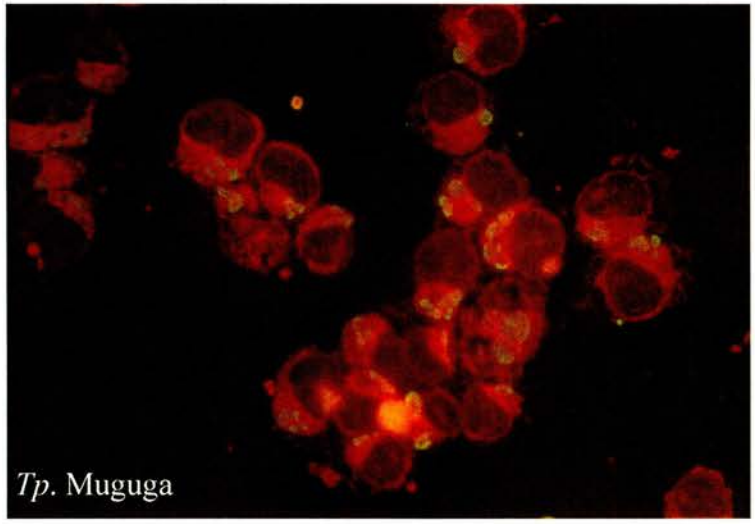
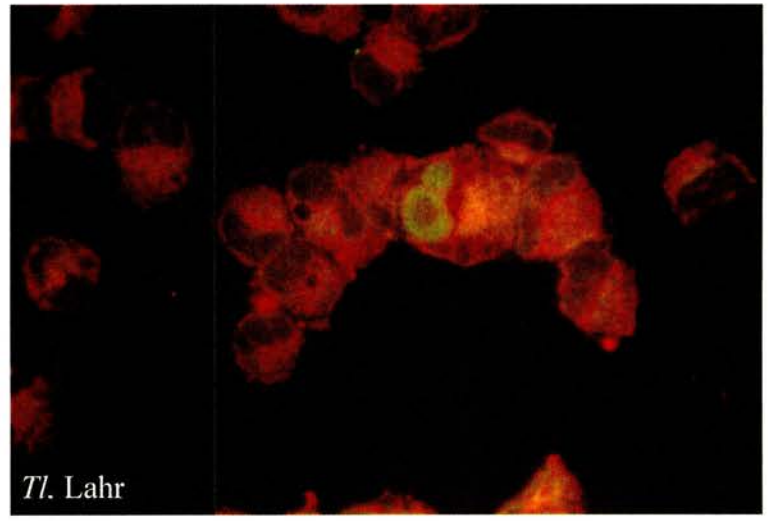
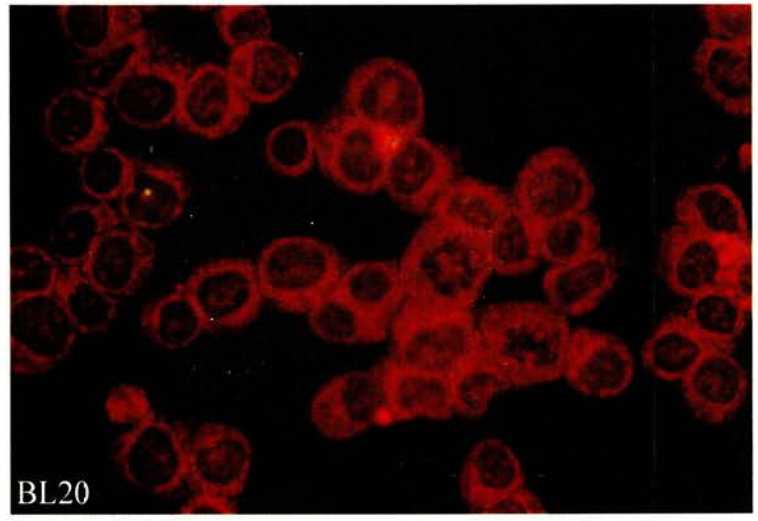

Ta. Hissar

Figure 4.21. continued


Tp. Muguga


Tl. Lahr


BL20

4.4. DISCUSSION

4.4.1. Screening of cDNA and Genomic DNA Libraries of *T. annulata*

The purpose of the current study was to define an immunogenic antigen from the macroschizont stage of *T. annulata* that could be used in enzyme linked immunoassays to detect stage-specific antibody responses against this parasite, especially in animals vaccinated with attenuated cell lines. Such an antigen could then be used in conjunction with the Tamr-1 antigen to distinguish vaccinated animals from those naturally infected in the field, since the Tamr-1 antigen has been shown to be detected by serum from animals infected with sporozoites and a low passage cell line but not animals vaccinated with a high passage cell line (Ilhan, 1995).

Initially, it was considered that the best approach would be to screen a genomic DNA expression library of *T. annulata* with sera from animals immunised with high passage cell lines. The basis of this approach was that such sera would recognise mainly macroschizont antigens, as it is generally accepted that the attenuated-high passage lines used for immunisation lose the ability to differentiate from macroschizonts to piroplasms (Pipano, 1977). However, most of the serum samples tested recognised not only macroschizont antigens but also piroplasm antigens including the 30/32 kDa major merozoite surface antigen (Tams1). Many of these proteins have not been shown to be present in the macroschizont stage, or are expressed at very low levels. Therefore, it is most likely that generation of merozoites has taken place in the animals vaccinated with the attenuated cell lines. Since neither merozoites nor piroplasms were observed in these animals, it is possible that macroschizonts of attenuated vaccine cell lines start to undergo differentiation, but are arrested during this process before viable merozoites are formed. An example of such a cell line is the Tova line which was maintained in the laboratory by serial passage through susceptible calves before isolated by cell culture and lost the capacity for the schizonts to develop into piroplasms but it was fully virulent (Pipano *et al.*, 1974). This cell line generates high levels of merozoite antigens *in vitro*, yet no morphologically-intact merozoites are observed (Shiels, unpublished observations). It is known that merozoite antigens can be produced relatively early during the differentiation process. For example the 30/32 kDa antigen

appears before free merozoites are produced *in vitro* (Shiels *et al.*, 1994). The other possibility is that macroschizont infected high passage cell lines produce merozoites and piroplasms at such low levels that they cannot be detected by blood smear examination, but are able to stimulate the immune system to produce anti-piroplasm antibodies. Taken together, the results precluded the use of sera from animals immunised with attenuated cell lines in screening a genomic DNA expression library of *T. annulata*.

The recognition of macroschizont antigens with serum samples from animals infected either with sporozoites or non-attenuated low passage cell lines was more prominent than that obtained using sera from animals immunised with attenuated cell lines. Serum samples from animals infected either with non-attenuated cell lines or sporozoites detected three major proteins, around 130 kDa, and 40 kDa that were specific to macroschizont extracts in western blot analysis. However, the use of these sera in screening a genomic DNA expression library of *T. annulata* was complicated since several piroplasm antigens were also recognised. When an anti-merozoite rabbit serum (C9m) was used to eliminate the detection of piroplasm antigens by immune bovine serum, only very few piroplasm antigens were eliminated, suggesting that the antiserum C9m did not react against all the piroplasm antigens within the extract. However, incubation of the immune bovine sera with crude piroplasm extracts effectively blocked the detection of most of the piroplasm and macroschizont antigens including the major 130 kDa macroschizont antigen. The 40 kDa macroschizont antigen could, however, still be detected strongly. This protein is likely to be the same antigen that was shown to be a polymorphic immunodominant molecule of *T. annulata,* which displayed variable mobility when examined from different isolates (Shiels *et al.*, 1994; Shiels per. comm.).

Initial screens of a genomic expression library using the immune bovine sera treated with piroplasm extracts were not successful in identifying a positive plaque. The reasons for this negative result are unclear. Screening expression libraries with an antibody can be difficult, as one has to rely on the assumption that the epitopes recognised by the serum will be replicated by the λgt11 expression system. Thus, it is possible that epitopes were not represented by the library due to either conformational changes or modification of the primary amino acid sequence

(Sambrook *et al.*, 1989), generating a false negative result. The immune bovine sera used in the current study recognised the 40 kDa antigen in western blot analysis. Therefore, it is unlikely that the failure to detect this protein was due to conformational changes (Sambrook *et al.*, 1989). Another possibility is that the antigen or the detected epitopes were not represented by the library before or after the induction of the gene expression.

Since attempts to clone the gene encoding the 40 kDa macroschizont antigen by immunoscreening the genomic expression library failed, an alternative strategy based on screening a cDNA library with a possible homologue of this gene was sought. The QP/PIM gene of *T. parva* was a good candidate for this purpose, for several reasons. First, both the 40 kDa protein (current study) and PIM (Toye *et al.*, 1991; Baylis *et al.*, 1993) are expressed by the macroschizont stage of the parasite. Second, both proteins are immunodominant. Third, both the 40 kDa protein (Shiels, pers.comm.) and PIM (Toye *et al.*, 1991; Toye *et al.*, 1995a) show size polymorphism when tested with different stocks of the parasite. Therefore, it is possible that the 40 kDa protein could be the *T. annulata* homologue of the QP/PIM antigen. In order to test if the *T. annulata* genome has a gene(s) homologue of the QP/PIM gene of *T. parva*, Southern blot analysis was performed. It was demonstrated that there are genes in the *T. annulata* genome that hybridised to the QP/PIM probe. These results were supported by northern blot analysis where the QP cDNA probe detected at least three mRNA species of *T. annulata*. On the basis of the preliminary data the QP probe was used to screen a cDNA library representing *T. annulata* macroschizont infected cells. Three main groups of clones, NC1, NC2, and NC10 were obtained.

4.4.2. Characterisation of Genes Isolated from the cDNA Library

In order to characterise cDNA clones NC1, NC2 and NC10, Southern blot, sequence and northern blot analyses were performed. Southern blot analysis of *T. annulata* genomic DNA using clones NC1, NC2 and NC10 as probes at low stringency revealed that the *T. annulata* genome contains several genes related to each of these clones (see Figures 4.6-4.8). Under high stringency conditions, a single restriction fragment was detected with digests of DNA from cloned macroschizont

infected cell lines probed with cDNA clones NC1 and NC10, but not with NC2. In some cases, however, multiple restriction fragments were obtained. Sequence analysis of clones NC1 and NC10, and restriction analysis of NC2 indicated that for certain enzymes there are multiple restriction sites within the corresponding genes. Multiple restriction fragments could also be due to partial digestion of genomic DNA. Based on detailed analysis of the obtain Southern profiles it was concluded that it is most likely that clones NC1, NC2 and NC10 represent single copy genes.

Southern blotting in conjunction with sequence analysis revealed that there is at least one intron in the NC10 gene with an estimated size of around 750 bp. The presence and size of this intron was confirmed by PCR (data not shown). Interestingly, this intron is much longer in length than introns of other genes of *Theileria* characterized to date. So far, only a total of 22 introns have been identified and their sizes varied between 29 and 159 bases in length (Nene *et al.*, 1998). Therefore, to the best of my knowledge, the intron in the NC10 gene is the longest intron found in *Theileria* to date. In addition, sequence analysis demonstrated that one of the cDNA clones in Group 1 (clone NC11) has an additional 33 bp insert that did not align with the consensus sequence of the NC1 gene. It is likely that this 33 bp insert is an unspliced intron as it contains splice site sequences (Shapiro and Senapathy, 1987).

The sequence analysis of the cDNA clone NC1 revealed an insert of 3.2 kb, which is smaller than the size of the mRNA species detected by northern blot analysis (around 3.6 kb). The longest ORF starts from nucleotide 691, making it an unusually long 5' untranslated region for *Theileria* (Katzer per. comm.). In addition, the ATG start codon at the beginning of this ORF is the 17th 'ATG' within the NC1 cDNA. It is unusual for the coding sequences of eukaryotic genes to be initiated with an ATG codon that does not correspond to the first ATG sequence in the transcript (Kozak, 1985). Since there is another ORF extending from nucleotide 1 to nucleotide 750, there could be a sequencing error between nucleotides 691 and 750 resulting in a frame shift in the ORF. If indeed there was a sequencing error, it is most likely that the sequence of the 5' end of the gene represented by clone NC1 is not complete, as the estimated size of the mRNA was larger than the insert. However, this possibility

could not be investigated due to time constraints and the sequence obtained will be verified by further studies.

As in the case of NC1, the size of the NC10 gene obtained by sequence analysis (5.4 kb) was smaller than the size of two mRNA species detected by northern blot analysis (6.2 and 7.4 kb). An ORF starts from nucleotide 480, indicating the presence of over 1 kb untranslated region at the 5' end. Since the sequence of the 5' end was obtained from genomic DNA, it is possibly that the ORF started downstream from the 3' end of an intron. If this is the case, then there is evidence for at least two introns in the NC10 gene, including the 750 bp intron. Additional studies are needed to obtain the complete cDNA sequence to verify the presence of introns, define the initiation ATG codon and determine which mRNA species is representative of the NC10 gene.

Both nucleotide and amino acid sequences of cDNA clones NC1, NC2 and NC10 were compared with the corresponding sequence of the gene encoding the QP/PIM protein. The region of similarity between the predicted amino acid sequences of the cDNA clones and the QP/PIM gene was found to be over the Gln-Pro and Glu-Pro regions. The sequence similarity between PIM and clones NC1, NC2 and NC10 was low. However, the NC10 gene shows tetrapeptide repeats with QP motifs in its central region. These repeats, in particular, are similar to the repeats observed in the PIM gene sequence (Baylis *et al.*, 1993; Toye *et al.*, 1995b). It has been demonstrated recently that similar motifs are also present in the p150 gene of *T. parva*, which immunologically cross reacts with PIM (Skilton *et al.*, 1998). In addition, like PIM, the p150 gene was also shown to be polymorphic (Skilton *et al.*, 1998). It was suggested that these motifs and sequence homologies may represent a common function or reflect a common ancestry for the p150 and PIM antigens (Skilton *et al.*, 1998). It is tempting to speculate that NC10 also shares a common ancestor with the p150 and PIM genes at some point in the past.

Northern blot analyses were performed to reveal the expression patterns of the cDNA clones. It was demonstrated that genes represented by clones NC1, NC2, and NC10 were differentially expressed by different life cycle stages of the parasite. The NC1 and NC2 genes were expressed mainly by the sporozoite stage and were clearly down-regulated at the piroplasm stage. However, it is difficult to conclude

that the down-regulation occurs during the macroschizont stage, since the amount of macroschizont RNA loaded was less than the sporozoite and piroplasm RNA due to the presence of bovine RNA in the samples obtained from infected cell lines.

In contrast, the NC10 gene was expressed mainly at the macroschizont stage of the parasite life cycle. Two mRNA species of 6.2 and 7.4 kb, were detected by the NC10 probe in northern blot analysis. Two hypotheses can be put forward for the presence of these two mRNA species. Firstly, if NC10 represent a single copy gene, two transcripts could be generated by the presence of different transcription initiation/termination sites, differential 3' processing and polyA addition, or by alternative RNA splicing as seen in other systems (Ranson *et al.*, 1998; Kitayama *et al.*, 1999). Complete sequence information and mapping of the 5' and 3' termini of the mRNA species by RACE analysis are needed to test this hypothesis. Secondly, although Southern blot analysis suggested that the NC10 is a single copy gene, these results are not conclusive as only a small number of restriction enzymes were tested. Therefore, additional related genes may be present in the genome as indicated by northern and western blot analyses and the results of IFAT with anti-NC10-Ssp13 sera.

The QP probe detected at least three *T. annulata* mRNA species. The size and the expression pattern of the mRNA species at about 3.3 kb obtained by the QP probe was similar to that obtained by NC1 gene. However, nucleotide sequence homology between the NC2 gene and the QP/PIM gene is low. At the stringency used a higher level of homology would be expected for detection of the same RNA species by these probes. Additionally, both QP and NC2 probes do not detect the same RFLP's on Southern blots at 60°C stringency. Therefore, either Southern or northern are different in terms of stringency or probes detect different mRNA species of approximately same size.

Taken together, none of the genes isolated from the *T. annulata* cDNA library appears to be the real homologue of the QP/PIM gene. However, it is possible that there is a gene(s) in the *T. annulata* genome with a higher homology to the QP/PIM gene, since Southern blot analysis of *T. annulata* Ankara piroplasm DNA with the QP probe showed that there are restriction fragments that are detected by QP probe following a high stringency wash (60°C). None of these restriction fragments were

detected by the NC1, NC2 or NC10 gene probes. Thus, these cDNAs may only have limited sequence relatedness to the QP probe. On the other hand, the NC10 gene is QP-like in terms of the tetrapeptide repeats with QP motifs and the encoded polypeptide could be a structurally related to and act as a functional homologue of the QP/PIM. Furthermore, as the QP/PIM gene is highly polymorphic among *T. parva* stocks (Toye *et al.*, 1995a; Toye *et al.*, 1995b), it is possible that the selection for diversity in this antigen in one or both species has resulted in the low level of homology displayed between PIM and the *T. annulata* genes cloned in this study.

4.4.3. Expression and Characterization of Proteins

Since both NC1 and NC10 genes were expressed by the macroschizont stage of the parasite, attempts were made to produce recombinant proteins from these genes. These proteins could then be used in a combination ELISA involving Tamr1 in an attempt to distinguish animals vaccinated with high passage cell lines from those naturally infected with *T. annulata*.

In order to express two fragments of the NC1 gene (1.2 and 2.2 kb in length), two expression constructs were designed using both pQE and pGex vector systems. It was demonstrated that expression products of both fragments were toxic to the strains of *E. coli* used, XL1 Blue and M15[pRep4]. It is possible that expression products of both fragments of the NC1 gene interfered with bacterial gene expression or other essential cellular processes (Spector *et al.*, 1998). Although various methods were used to overcome the lack of expression due to toxicity, the purified expression product obtained from the 1.2 kb fragment of the NC1 gene in the pQE system was not sufficient enough for further studies. In addition, the His-tagged fusion protein did not react with the immune bovine serum. This indicated that the fusion protein was not immunogenic and, therefore, could not be used in an ELISA.

In order to express a fragment of the NC10 gene, three different strategies were followed. Expression of the whole cDNA fragment using the pGex vector resulted in very low levels of expression product, and no expression products were obtained from a fragment spanning the QP repeat region of the NC10 gene. To overcome the lack of expression, smaller fragments were obtained using several restriction enzymes that recognised multiple sites in the cDNA sequence. The

expression of these fragments resulted in several GST fusion proteins. The expression product of the NC10-Ssp13 fragment was more immunogenic than the other GST fusion proteins, making it a good candidate to use in ELISA. The use of a GST fusion protein in ELISA, however, could be complicated since GST antigens are present in *Schistosoma bovis* and *Fasciola hepatica* (Hillyer *et al.*, 1992), and infections with these parasites occur in areas where tropical theileriosis is endemic (Kaplan, 1994; Gargili *et al.*, 1999). Although previous studies indicated that serum samples from animals infected with *S. bovis* and *F. hepatica* did not cross react with PIM GST fusion protein in an ELISA (Katende *et al.*, 1998), it was necessary to rule out the possibility of false-positive findings due to the GST. For this purpose, the Ssp13 fragment of the NC10 gene was expressed in a pQE vector as His-tagged fusion protein.

For the NC10-Ssp13 His-tagged fusion protein, the molecular mass predicted from the protein sequence is less than that estimated from polyacrylamide gel electrophoresis. This discrepancy may be due to the presence of sequences of alternating proline residues, particularly Glu-Pro and Gln-Pro, which is thought to confer an elongated conformation on proteins (Brewer *et al.*, 1990). This conformation would retard the migration of the NC10-Ssp13 fusion protein in a polyacrylamide gel, as in the case of other proline-rich proteins (Carroll and Laughon, 1987).

As indicated above, the expression product of the NC10-Ssp13 fragment appeared to be more immunogenic than the other GST fusion proteins. This region of the gene included approximately a third of the repeated amino acid sequence. It has been demonstrated in many other organisms that repeated regions of amino acid sequences are immunogenic (Schofield, 1991). For example, in *Plasmodium spp*, antibodies to sporozoites appear to be directed almost entirely towards the repetitive domain of the circumsporozoite (CS) protein (Zavala *et al.*, 1985). Similarly, studies carried out with monoclonal antibodies against the PIM protein indicated that the repeat region of this molecule contains immunogenic epitopes (Toye *et al.*, 1996). Therefore, it is likely that the repeats within the NC10-Ssp13 fragment are responsible for a major proportion of its immunogenicity.

In order to confirm the antigenicity of the recombinant NC10-Ssp13 protein and to obtain more information about the native protein, western blot analyses were performed using an antiserum raised against the His-tagged NC10-Ssp13 fusion protein. Results of western blotting demonstrated that several polypeptides from macroschizont infected cells of different *T. annulata* stocks reacted with the rabbit anti-NC10-Ssp13 serum. Two explanations can be put forward for this observation. First, since the NC10-Ssp13 protein contains repeated amino acid motifs, it is possible that some of the bands were due to the cross reactivity of the anti-serum with similar repeats within different proteins. This possibility was shown previously for malaria antigens where cross-reactions occurs between epitopes in repeats of different proteins such as circumsporozoite (CS) proteins and asexual blood-stage membrane antigen (CRA) (Anders, 1986). Furthermore, it was shown that anti-PIM mAb 8 reacted with both recombinant PIM and recombinant p150 of *T. parva*. A sequence that occurs twice in the repeated region of the p150 has homology with the PIM peptide sequence that contains the mAb 8 epitope (Skilton *et al.*, 1998). There were no other regions of sequence with comparable homology between p150 and PIM. It is entirely possible therefore that the repeated epitopes in NC10-Ssp13 are present in other *T. annulata* macroschizont antigens. The detection of several related genes in Southern blotting, at reduced stringency, of the *T. annulata* genome using the NC10 cDNA probe supports of this hypothesis. The second possibility is that the detection of several polypeptides is due to the presence of more than one related gene for NC10 in the *T. annulata* genome. This is supported by two observations: (i) Two mRNA species were detected by the NC10 cDNA probe in northern blot analysis at high stringency which could suggest that there are two genes for NC10. Therefore, it is possible that the two large polypeptides detected in western blot analysis are derived from these two mRNA species and that the remaining polypeptide bands could be due to breakdown or processing products of these proteins; (ii) IFAT results showed that the anti-NC10 sera detected protein(s) on both macroschizonts and nucleus of the host cells. Clearly, further Southern blot analysis carried out at high stringency is necessary to distinguish between these two possibilities as the current data indicating the existence of a single copy NC10 gene requires confirmation.

On analysis by immunofluorescence microscopy, reactivity of NC10-Ssp13 anti-serum was detected against both macroschizonts and the host cell nucleus. Host nuclear reactivity was scattered over the region of the nuclear envelope and appeared to be absent from the nucleoli. A similar pattern of reactivity was observed among different stocks of *T. annulata* including the BL20 cell line infected with *T. annulata* Ankara, while the uninfected lymphoblastoid BL20 cell line was negative. These results indicate that the protein detected on the host nucleus may be related to infection and immortalisation of the host cell by the parasite. However, with the data available, cross recognition of a host polypeptide cannot be discounted. It is also possible that a parasite protein transported to the nucleus is not encoded by the NC10 gene as Southern, northern and western blotting suggest there may be related genes in the *T. annulata* genome and/or cross recognition of polypeptides with related repeat epitopes.

Variations were observed in the level of reactivity against the host nucleus both between and within the macroschizont infected cell lines that were tested. This could be due to the presence of different parasite populations in the *in vitro* established cell lines as it is known that they can represent a number of distinct parasite genotypes. It is possible, therefore, that one polypeptide or different forms of the same polypeptide could be polymorphic across different parasite populations. However, variations were also obtained within cell lines (D7 and D7B12), which are thought to represent the same genotype, suggesting that the intensity of staining could be related to the expression level of the same protein(s) and it is known that alterations to gene expression that result from adaptation to *in vitro* cultivation might also lead to variation in the intensity of staining (Baylis *et al.*, 1992; Shiels *et al.*, 1994; Sutherland *et al.*, 1996).

The staining of the host cell nucleus appeared to be specific to cells in interphase as no staining was observed in cells visibly undergoing mitotic division. In contrast, macroschizonts were detected during all cell cycle stages. Detection of reactivity on the host nucleus during interphase and its absence during mitosis would suggest that loss of reactivity could be related to the disintegration of the nuclear envelope, i.e. the protein could be attached or inserted into the nuclear membrane, and this possibility is supported by the observation of fluorescence in the cytoplasm

of mitotic cells. Nevertheless, it is not possible to reach a definitive conclusion from the IFAT pattern alone.

A molecule that may be of parasite origin and is located to the host cell nucleus is clearly of interest with respect to the parasite induced immortalisation of the host cell. At present, identification of mechanisms related to induction of host cell proliferation by *Theileria* is limited and not fully understood (reviewed by Chaussepied and Langsley, 1996; Dobbelaere and Heussler, 1999). It has been demonstrated that host cell division depends on the presence of the macroschizont, as drug-induced death of the parasite results in termination of proliferation and induction of apoptotic host cell death (McHardy and Morgan, 1985; Dobbelaere *et al.*, 1988; Fich *et al.*, 1998). Several studies have demonstrated changes in important host cell regulatory molecules in *Theileria*-infected cells; such as the transcription factors NFκK (Ivanov *et al.*, 1989); AP-1 (Baylis *et al.*, 1995); interleukin 2 (IL-2) and the IL-2 receptor (IL-2R) (Dobbelaere *et al.*, 1988; Heussler *et al.*, 1992) and Casein kinase II (Ole Moiyoi *et al.*, 1993). However, it is not known how the infection of the host cell by the parasite initiates these changes associated with immortalisation infection. The first candidate for a parasite molecule that is transported to the host cell nucleus and could be involved in regulating proliferation and/or host cell gene expression was reported recently (Swan *et al.*, 1999). This molecule (TashAT) has homology over a small motif (the AT hook) which is known to bind to A.T rich DNA and is present in molecules with known oncogenic potential (Tkachuk *et al.*, 1992; Ashar *et al.*, 1995; Slany *et al.*, 1998). Although the pattern of staining detected by anti TashAT sera and anti NC10-Ssp13 sera are related, they are not identical. Staining with anti TashAT antibody, for example, has been observed in cells during mitosis (Shiels, Stern and Swan, unpublished). Thus while the TashAT genes and NC10 may both be involved in the control of host cell division/gene expression it is possible they perform distinct complementary functions related to a location either within or on the surface of the host nucleus.

The pattern of reactivity in actively dividing cells with anti NC10-Ssp13 antibody is interesting as it mimics the pattern observed for nuclear envelope polypeptides especially the lamina protein, (LAP) 2α (Dechat *et al.*, 1998). The nuclear envelope forms the boundary of the nucleus in eukaryotic cells and separates

nuclear and cytoplasmic activities. It consists of a double membrane, an underlying filamentous meshwork (the nuclear lamina) and nuclear pore complexes that mediate nucleo-cytoplasmic transport. The structural integrity of the nuclear envelope is essential for nuclear functions such as DNA replication, RNA transcription and RNA processing (Dechat *et al.*, 1998). In higher eukaryotes the nuclear envelope breaks down during mitosis (open mitosis) and the nuclear lamina is transiently disassembled, most probably through phosphorylation of lamina proteins by protein kinases which are key regulators of the cell cycle (Kendrew and Lawrence, 1994). By altering the nuclear envelope structure or nuclear pore complexes, essential replication factors may be enabled to cross the nuclear envelope to enter the nucleus and to start DNA replication (Coverley *et al.*, 1993). Thus, it is possible that a parasite polypeptide associated with host nuclear envelope may function to alter the envelope structure or nuclear pore facilitating the transport of molecules involved in regulating host nuclear function, as proposed for the TashAT polypeptides (Swan *et al.*, 1999).

Evidence for a putative transport structure has been described previously in *T. annulata* infected cells. Thus, Musisi *et al.* (1981) and Jura *et al.* (1983a) demonstrated the possibility of a connection between the host cell nucleus and the macroschizont via annulate lamellae at interphase of the cell cycle by electronmicroscopy, but no conclusion could be made as to the precise nature of this connection. These membranes are often observed in rapidly dividing cells, germ cells, and cells at the onset of differentiation (Kessel, 1992). The function of this membrane system in these cells is yet to be elucidated. Recently, studies on lymphoid cells infected by human herpesvirus 6 using anti-endoplasmic reticulum antibodies showed a close relationship between annulate lamellae and the endoplasmic reticulum and nuclear membranes (Cardinali *et al.*, 1998). In addition, at least some of the proteins involved in the nuclear pore complex formation have common binding properties with proteins of the annulate lamellae (Cordes *et al.*, 1995). From these findings it is conceivable that annulate lamellae are involved in the transport of molecules to the nucleus of proliferating cells and may contain the NC10 polypeptide in *Theileria* infected cells.

In order to determine whether the NC10 antigen has a structural role, a number of studies need to be performed. These include immunoelectron microscopy during different phases of the cell cycle. Such studies could provide further information on the fate of the antigen during mitosis. Is it processed, for example, or could it be associated with the microtubule network involved in nuclear assembly? A role for these functions has been described for lamin B proteins (Ellenberg *et al.*, 1997). Is the molecule associated with the schizont present on the parasite surface and does it allow attachment to the host mitotic spindle? Transfection studies, as described for the TashAT gene (Swan *et al.*, 1999), should also be performed. Importantly, they will confirm whether the NC10 antigen is responsible for the fluorescence pattern described in this study or whether this is due to a related parasite antigen or cross recognition of a host polypeptide, although the lack of reactivity in a range of host nuclei including BL20 and *T. parva* infected cells suggests this is unlikely.

The aim of the current study was to define an immunogenic antigen from the macroschizont stage of *T. annulata* that could be used in ELISA to detect antibodies against this parasite in both animals vaccinated with high passage cell lines and those that are naturally infected. To facilitate this goal, recombinant proteins encoded by genes expressed at the macroschizont stage of the parasite were obtained. Among these, the NC10-Ssp13 fusion protein was found to be immunogenic, making it a good candidate for ELISA. Results of experiments aimed at development of an indirect ELISA using recombinant NC10-Ssp13 protein in combination with the recombinant merozoite rhoptry antigen (Tamr-1) and a second macroschizont antigen (Tash-2) are described in Chapters 5 and 6.

CHAPTER FIVE

DEVELOPMENT OF INDIRECT ENZYME LINKED IMMUNOSORBENT ASSAYS USING RHOPTRY AND MACROSCHIZONT ANTIGENS

5.1. INTRODUCTION

The collection of serum samples and production of the macroschizont stage specific recombinant antigen for the enzyme linked immunosorbent assay (ELISA) were described in Chapters 3 and 4, respectively. This chapter focuses on the use of the stage specific recombinant antigens in serological tests.

Detection of antibodies specific to *T. annulata* is necessary not only to assess the prevalence of tropical theileriosis in epidemiological studies but also to measure the impact of control programmes for *T. annulata* in endemic areas. To date, the indirect fluorescent antibody test (IFAT) using both macroschizont and piroplasm antigens has been extensively used for the detection of antibodies against *T. annulata* in cattle following infection (Pipano and Cahana, 1969) and vaccination with attenuated cell lines using macroschizont antigen (Pipano *et al.*, 1969). However, the IFAT fails to distinguish vaccinated/unchallenged animals from those that are either vaccinated/challenged or those that are naturally infected by ticks due to the presence of common antigens in all stages of the parasite (Knight *et al.*, 1998). Such distinction is important to assess the efficacy of a vaccine against possible challenge(s) in the field.

ELISA has several advantages over the IFAT. For example, IFAT relies on subjective observation of degree of fluorescence whereas ELISA provides objective results. ELISA systems allow large numbers of samples to be assayed. In addition, the potential sensitivity of ELISA is greater than IFAT. As a result, several attempts have been made to develop an ELISA to detect antibodies specific to *T. annulata* (Gray *et al.*, 1980; Kachani *et al.*, 1992; Sundar *et al.*, 1993; Reddy *et al.*, 1994; Beniwal *et al.*, 1997). In these studies, crude antigen extracts obtained from piroplasms (Gray *et al.*, 1980; Sundar *et al.*, 1993), macroschizont (Reddy *et al.*, 1994; Prasanth *et al.*, 1995) or both macroschizont and piroplasm (Kachani *et al.*,

1992; Beniwal *et al.*, 1997) stages of the parasite were used. However, none of these tests were fully evaluated for sensitivity and specificity. In addition, it has been demonstrated that ELISAs performed with crude antigen extract from the piroplasm stage of the parasite cannot distinguish between vaccinated and naturally infected animals (Kachani *et al.*, 1996; Beniwal *et al.*, 1997).

One approach to increase sensitivity and specificity of the test would be to use recombinant antigens rather than crude piroplasm antigen preparation. Furthermore, if the recombinant antigen used is expressed in a specific stage of the parasite, it would be possible to identify the stage of the parasite that an animal has been exposed to. Such a test could then be used to distinguish vaccinated animals from those that are naturally infected. Following a natural infection with *T. annulata* sporozoites, antibodies would be detected against sporozoites, macroschzionts, merozoites and piroplasms. In contrast, animals immunised with attenuated high passage cell lines, which do not produce piroplasms, will be exposed only to the macroschizont stage of the parasite. Therefore, serological tests using antigens specific to the sporozoite or piroplasm stages of the parasite could only detect animals infected with sporozoites, and thereby distinguish these animals from vaccinated cattle. On the other hand, tests performed with macroschizont-specific antigens would detect both naturally infected and vaccinated animals.

It is evident from the above discussion that identification of recombinant antigens specific to sporozoites of *T. annulata* would be of pivotal importance in distinguishing vaccinated animals from naturally infected ones. Previous studies performed with a sporozoite stage specific recombinant antigen, SPAG-1, showed that the SPAG-1 ELISA is not sensitive enough to detect animals exposed to sporozoites only once (Matita, 1994; Williamson *et al.*, 1994) as this is a regular occurrence in the field only once or twice in a year (Flach and Ouhelli, 1992). This could be due to the fact that the immune system is exposed to sporozoites for a very short time period before sporozoites enter the host lymphocytes and differentiate to macroschizonts (Jura *et al.*, 1983a).

An ELISA based on an antigen specific to the merozoite / piroplasm stage of *T. annulata* would detect naturally-infected but not vaccinated animals, provided that attenuated cell line vaccines do not produce piroplasms. An indirect ELISA using a

merozoite surface antigen, Tams1-1, was recently developed by Gubbels *et al.* (1999a). Their study demonstrated that the Tams1-1 ELISA is not sufficiently sensitive to detect animals infected with sporozoites, when the test is performed earlier than three months post infection (Gubbels *et al.*, 1999a). In addition, a 30-32 kDa merozoite surface antigen (Tams1) was detected in Western-blot analyses using serum samples from animals immunised with the *T. annulata* Ankara/Pendik attenuated cell line (Chapter 4). Thus, it is possible that the Tams1-1 ELISA would detect not only naturally infected animals but also those that are vaccinated with attenuated cell lines.

A recombinant merozoite rhoptry protein, Tamr-1, is another antigen specific to the piroplasm stage of *T. annulata*. Preliminary studies carried out by Matita (1994) demonstrated that Tamr-1 is antigenic. In a further preliminary study, it was found that when animals are infected with sporozoites or a low passage cell line they showed antibody responses in the Tamr-1 ELISA (Ilhan, 1995). In contrast, animals immunised with attenuated high passage cell lines showed no or only a slight increase in antibody levels (Ilhan, 1995). These studies suggested that Tamr-1 could be used in an ELISA designed to distinguish between naturally infected and vaccinated animals. However, the diagnostic sensitivity and specificity of the Tamr-1 ELISA remain to be evaluated.

While Tamr-1 ELISA could be used to detect naturally infected animals, an ELISA that could detect antibodies in vaccinated animals is also needed to verify that macroschizonts are established in the recipient animal. This could be accomplished using an antigen expressed in the macroschizont stage of the parasite, such as NC10-Ssp13 (see Chapter 4). An antigen that is expressed in both the macroschizont and piroplasm stages of the parasite such as Tash-2, which was shown to be immunogenic (D. Swan, pers. comm.), could also be used for the same purpose.

In the current study, the three recombinant antigens, Tamr-1, NC10-Ssp13 and Tash-2 were evaluated by ELISA using guidelines for the international standardisation of antibody–detection ELISA protocols (Wright *et al.*, 1993). The objectives of this chapter were: (i) to determine the immunogenicity of Tamr-1, NC10-Ssp13 and Tash-2 against immune bovine sera using Western blot analysis; ii) to standardise working protocols for indirect IgG and IgM ELISA using Tamr-1,

NC10-Ssp13 and Tash-2; iii) to establish the diagnostic sensitivity and specificity of these ELISAs. In the following chapter (Chapter 6), these antigens will be further analysed for the purpose of distinguishing vaccinated animals from those that are infected by ticks in the field.

5.2. MATERIALS AND METHODS

5.2.1. Purification of Recombinant Antigens

5.2.1.1. Merozoite rhoptry antigen (Tamr-1)

The merozoite rhoptry (Tamr-1) recombinant protein was expressed as a GST-E21X fusion protein using the pGex-1N gene fusion vector (Pharmacia). The clone containing two-thirds of the rhoptry gene was kindly provided by Dr. Brian Shiels (Department of Veterinary Parasitology, University of Glasgow). The recombinant protein was purified as described in section 4.2.20.2 and the quality of the protein was assessed by 10% SDS-PAGE (see section 4.2.2). The protein was reconstituted in PBS at a concentration of 3.5 mg/ml. The fusion protein was divided into 50 µl aliquots and stored at –70°C.

5.2.1.2. NC10-Ssp13 antigen

The macroschizont NC10-Ssp13 recombinant antigen was expressed in pQE-32 vector as a His_6-tagged protein. The recombinant protein was purified as described in section 4.2.20.1 and was reconstituted in Tris-HCl, pH 8.0, at a concentration of 160 µg/ml.

5.2.1.3. Tash-2 antigen

The recombinant Tash-2 protein was kindly provided by Dr. David Swan (Department of Veterinary Parasitology, University of Glasgow) as a GST fusion protein. The concentration of recombinant protein was 130 µg/ml in PBS.

5.2.2. Western Blot Analysis of Recombinant Proteins

Western blot analysis was carried out using serum samples from animals infected with either sporozoites or low or high passage cell lines as described in section 4.2.3. The standardisation of protein concentrations, serum and conjugate dilutions was carried out by serial dilution of each recombinant protein. For Western blot analysis, 100 ng of recombinant protein was loaded to each lane in SDS-PAGE loading buffer. Bovine sera were diluted at 1:500 and alkaline phosphatase conjugated secondary antibody raised in rabbit (IgG whole molecule, Sigma, A-0705) was diluted at 1:20,000 in blocking buffer. Since both Tamr-1 and Tash-2 antigens were expressed as GST- fusion protein, serum samples were also tested against glutathione-S-transferase (GST), at 29 kDa. For this purpose, 100 ng of the GST protein was loaded on respective lanes.

5.2.3. ELISA

5.2.3.1. Theileria annulata *antibody ELISA reagents and protocol*

The indirect-ELISA protocol was used to detect both IgG and IgM antibody responses of animals using Tamr-1, NC10-Ssp13 and Tash-2 recombinant antigens.

i) Reference control sera

A set of pre- and post infection serum samples was tested to choose the negative control (C-), low positive (C+) and high positive (C++) reference control sera to be included as standard control sera in future tests. For IgG ELISAs, serum samples were tested using 1:25-1:3,200 antigen dilution, 1:50 serum dilution and 1:5,000 conjugate dilution. Dilutions for IgM biotin conjugate (immunoglobulin fraction of mouse ascites fluid, clone BM-23) (Sigma; B-0774) and the ExtrAvidin® (Sigma; E2886) were 1:10,000 and 1:1,000, respectively. Where applicable, pre-infection serum samples with an optical density (OD) reading of around 0.1-0.2 were used as negative (C-) control sera. Serum samples with OD readings of 0.5-0.7 and 1.0-1.5 were used as low positive (C+) and high positive (C++) control sera, respectively. Selected negative (C-), low positive (C+) and high-positive (C++) control sera were aliquoted into 500 µl microfuge tubes and stored at –20°C until use.

ii) Conjugate

Affinity purified, rabbit anti-bovine IgG horseradish peroxidase-labelled conjugate (HRPO) developed in rabbit (Sigma; A-5295) was used for IgG ELISAs. The conjugate was stored at –20°C in 50 µl aliquots. Once thawed, the conjugate was kept at + 4°C. An anti-bovine IgM biotin conjugate (immunoglobulin fraction of mouse ascites fluid, clone BM-23) (Sigma; B-0774) was used with ExtrAvidin®-peroxidase (Sigma; E2886) for IgM ELISAs.

iii) Coating buffer

Carbonate-bicarbonate buffer, pH 9.6, 0.05M, (Sigma) was prepared by dissolving one capsule in 100 ml deionised water. The buffer was kept at 4°C and used within one week.

iv) Washing buffer

Phosphate-buffered saline (PBS, 144 mM Sodium chloride, 1.47 mM Potassium dihydrogen orthophosphate, 8.1 mM di-Sodium hydrogen orthophosphate dodecahydrate, 2.68 mM Potassium chloride, pH 7.4), with 0.05% polyoxyxyethylene sorbitan monolaurate (Tween 20) (PBST/0.05T), was prepared and kept at room temperature for up to four weeks.

v) Serum and conjugate diluents

Phosphate-buffered saline, pH 7.4, with 0.5% Tween 20 was used as diluent for all the ELISAs. It was kept at room temperature for up to two weeks.

vi) Substrate buffer

One component Tetramethylbenzidine dihydrochloride (TMB) Microwell Peroxidase Substrate (Kirkegaard and Perry Laboratories) was used according to the manufacturer's directions.

5.2.3.2. Basic ELISA protocol

i) Detection of immunoglobulin G antibodies

All ELISAs were performed using 96 well polystyrene micro-ELISA plates (Dynatech, Immulon, Type I). Antigen prepared in carbonate-bicarbonate buffer was added at 100 µl per well and plates were covered with plate sealers (ICN Biomedicals). Plates were put on a microplate incubator/shaker (Dynatech Laboratories Ltd) for 30 seconds at room temperature to obtain an even distribution of the solution at the bottom of each well. Plates were then kept overnight at 4°C. Each well was washed five times with 300 µl PBS/0.05%T using a Multichannel Dispenser Pipette (Finnpipette). The content of each well was removed by shaking out, and plates were blotted upside down on paper towels to remove excess buffer. Test samples and control sera were diluted in PBS/ 0.5%T and added, 100 µl per well, in duplicate and quadruplicate wells, respectively. Four controls were included on each plate: the conjugate control (Cc) which was serum diluent only, high positive (C++), low positive (C+), and negative (C-) controls. The plates were covered with plate lids and incubated at 37°C for 1 hour on the microplate shaker. Following the incubation, plates were washed with PBS/0.05%T as described above. The horseradish peroxidase conjugate diluted with PBS/0.5%T was added at 100 µl into each well. Plates were covered, incubated for a further one-hour period in the microplate shaker and washed with PBS/ 0.05%T. One hundred microlitres of the TMB substrate solution was added into each well and plates were incubated at room temperature for 10 minutes while shaking. The enzymatic reaction was stopped by adding 100 µl of 0.2 M sulphuric acid per well. The ODs of individual wells were read immediately using a Labsystems Multiskan Plus II ELISA plate reader (Life Sciences International, UK, Ltd.) with a 450 nm filter. Before reading the test results, the plate reader was blanked against an empty ELISA plate of the same type used for the test.

ii) Detection of immunoglobulin M antibodies

ELISA for detecting IgM was performed as described in section 5.2.3.2(i) with slight modifications. Incubation periods for coating plates, diluents, washing

buffer, incubation times, temperatures, and volumes of the samples were the same as for IgG ELISAs. Biotin labelled anti-bovine IgM monoclonal antibody was diluted with PBS/0.5%T and 100 µl of antibody solution was added into each well following the incubation serum samples. Following incubation and washing steps, 100µl ExtrAvidin®-peroxidase conjugate diluted in PBS/0.5%T was added into each well. Plates were incubated at 37°C for 45 minutes. The colour development and measurement was as described above for IgG ELISAs.

5.2.3.3. Standardisation and optimisation of indirect ELISAs for the detection of T. annulata *specific antibodies*

Chequerboard titrations were conducted to determine which antigen, serum and conjugate dilutions gave optimal differentiation between OD readings of positive (C++) and negative sera (C-). For this purpose, the ratio of positive and negative control ODs (OD_{C++}/ OD_{C-}) was calculated as described previously (Voller *et al.*, 1976).

5.2.3.4. Data expression and quality control parameters

The data obtained from the IgG Tamr-1 ELISA assays were expressed according to the guidelines for the international standardisation of antibody detection ELISA protocols and reagents (Wright *et al.*, 1993). The data were expressed as percentage positivity (PP) as described by Wright *et al.* (1993). The PP value of the OD of each well was calculated using the following equation:

$$PP = \frac{\text{Single well OD}}{\text{Median } OD_{C++}} \times 100$$

where the median OD_{C++} equals the median of at least three high positive control ODs which were within the upper (UCL) and lower (LCL) control limits.

The OD and PP values of the high-positive control (C++), and the PP values of the low-positive (C+), negative (C-) and conjugate (Cc) controls were used to determine whether each test plate was within acceptable limits of variability, as defined by the upper (UCL) and lower (LCL) control limits. UCL and LCL were

determined for the reference control serum samples (C++, C+, C- and Cc) of the IgG Tamr-1 ELISA. In order to establish these limits, four plates with multiple replicate wells for each control were assayed on different days by the IgG Tamr-1 ELISA, under as near to ideal condition as possible. New plasticware and exact incubation times were used for each assay. The high-positive control (C++) was added to column 1-3, the low-positive control to columns 4-6, the negative control (C-) to column 7-9, and the conjugate control (Cc) to column 10-12.

The lower and upper control limits for the Tamr-1 ELISA controls (C++, C+, C-, Cc) were determined by the 90th and 10th percentiles of the replicate well OD and PP values, respectively (A.G. Luckins, per. comm.). Because the data did not have a Normal distribution when measured by the correlation coefficient using Minitab software (version 12), the percentile was obtained by sorting the data in descending order.

Individual test plates were accepted or rejected using three criteria. First, at least three of the four replicate high-positive control (C++) ODs had to be within the established UCL and LCL. Second, at least three of the four replicate PP values for each control (C+, C- and Cc) had to be within their respective upper and lower control limits. Third, the difference between ODs of replica test samples had to be less than 15%.

Data for IgM Tamr-1, NC10-Ssp13 and Tash-2 ELISAs were expressed as optical densities (OD). LCL and UCL were not determined for these ELISAs due to time constraints. In these ELISAs the antibody response to infection with *T. annulata* was monitored in each individual animal. In order to avoid plate to plate variations, sequential serum samples obtained from each animal were tested on the same ELISA plate. Antibody responses were correlated with the corresponding pre-infection values.

5.2.3.5. Estimation of sensitivity and specificity and cut-off analysis

Known positive serum samples from British cattle (n=209) experimentally infected with eight different stocks of *T. annulata* (Ankara, Diyarbakir, Gharb, Hissar, Ode, Tova, Tunisia, Razi) were used to determine the sensitivity of the IgG Tamr-1 ELISA. These sera were generated at the CTVM over the past 15-20 years.

Serum samples were taken from animals 28 to 58 days after a single infection with sporozoites or macroschizont- infected low and high passage cell lines of *T. annulata*. All samples have been tested by IFAT using both the schizont and piroplasm antigens. All the serum samples chosen for the sensitivity test were positive in the piroplasm IFAT at a 1:160 cut-off value. Known negative serum samples (n= 188) from pre-infection experimental calves were tested to determine specificity of the IgG Tamr-1 ELISA.

Cross reactions with other blood protozoans were tested by sera from cattle experimentally infected with *T. parva* (n=9 from CTVM; Muguga, Marikebuni, Lanet and Boleni stocks), *T. buffeli* (n=13; 2 from Australia, 11 from Sicily - serum samples from Sicily were collected from cattle infected in the field), *T. sergenti* (n=2 from Japan; Ikeda and Chitose stocks), *Babesia bovis* (n=12 from CTVM and ILRI, Mexico stocks), *B. bigemina* (n=9 from CTVM and ILRI, Muguga and Zaria stocks), *Anaplasma marginale* (n=8 from ILRI) and *Trypanosoma evansi* (n=2 from CTVM, Indonesian isolate TREU 1994, kindly provided by Dr. Tony Luckins). Serum samples from three Scottish cattle infected with the nematode *Ostertagia ostertagi* were also tested to see if anti-GST antibodies in these animals would cross react with the Tamr-1 antigen.

Optical density (OD) values were transformed to PP values as described in section 5.2.1.5. In order to choose optimal ELISA cut-off values, two-graph response operating characteristic curves (TG-ROC), which are equivalent to cumulative frequency distribution of negative (CFD_{neg}) and the inverse positive (CFD_{pos}) sample (Greiner *et al.*, 1995), were established using cut-off values of 0 - 100 PP at 1 PP intervals for the IgG Tamr-1 ELISA.

Positive and negative threshold PP values were chosen arbitrarily to obtain the optimal sensitivity and specificity for the IgG Tamr-1 ELISA. Test sensitivity was calculated as the number of the test positive animals divided by the total number of *T. annulata*-infected animals tested, and was expressed as percentage. Test specificity was calculated as the number of test-negative animals divided by the total number of non-exposed animals tested, and was expressed as percentage.

5.2.3.6. Blind test

A set of 240 serum samples (re-coded) selected by Dr. Susanna Williamson from experimentally infected animals served as "Gold Standard" samples according to the international standardisation and validation of different ELISAs for bovine tropical theileriosis. There were 86 negative serum samples from Scottish calves (pre-infection), 144 positive (post infection and post challenge) and 10 serum samples from animals which were infected with various blood protozoa (n= 6 *T. parva*, n=1 *T. buffeli*, n= 1 *B. bigemina*, n=1 *B. bovis*). Results from the IFAT using the piroplasm antigen were available for most of these samples. The presence of fluorescence at a dilution of 1:160 and above was accepted as positive. These serum samples were aliquoted in 250 µl volumes and distributed to a laboratory in Utrecht and to another laboratory in Morocco. The blind test that was performed at the CTVM was conducted using the IgG Tamr-1 ELISA, whereas the one at the Utrecht laboratory was conducted using a 32 kDa merozoite surface antigen (Tams1) ELISA. A blind test is yet to be performed in Morocco. Although exchange of antigens used in ELISAs between the two laboratories, *viz.* CTVM and Utrecht Laboratory, was planned, this could not be achieved due to time constraints.

5.2.4. Statistical Analysis

For each control serum sample, the edge-effect factor between inner and outer wells in the repeated quality control limits was analysed by Mann-Whitney test using Minitab (version 10.2). Confidence intervals (CI) for sensitivity and specificity of the IgG Tamr-1 ELISA were calculated as described by Thrusfield (1995). Differences between estimates of sensitivity were assessed by the Chi-square distribution using Minitab (version 10.2). When expected counts were less than five, the Fisher's correct test was employed instead of the Chi-square test using Instat statistic. The data were analysed for significance at the 5% level. The *kappa* statistic was used to assess the agreement between the Tamr-1 ELISA and piroplasm IFAT (Thrusfield, 1995).

5.3. RESULTS

5.3.1. Western Blot Analysis of the Recombinant Antigens

Results of Western blot analysis of a set of serum samples from animals infected with different stocks (Ankara, Hissar, Gharb) and life cycle stages (sporozoite or macroschizont) of *T. annulata* are given in Table 5.1. Figures 5.2A, B and C show results of representative Western blots of the Tamr-1, NC10-Ssp13 and Tash-2 antigens tested with these sera.

Table 5.1. Western blot analysis of a set of serum samples from animals infected with different stocks (Ankara, Hissar, Gharb) and life cycle stages (sporozoite or macroschizont) of *T. annulata* using Tamr-1, NC10-Ssp13 and Tash-2 antigens.

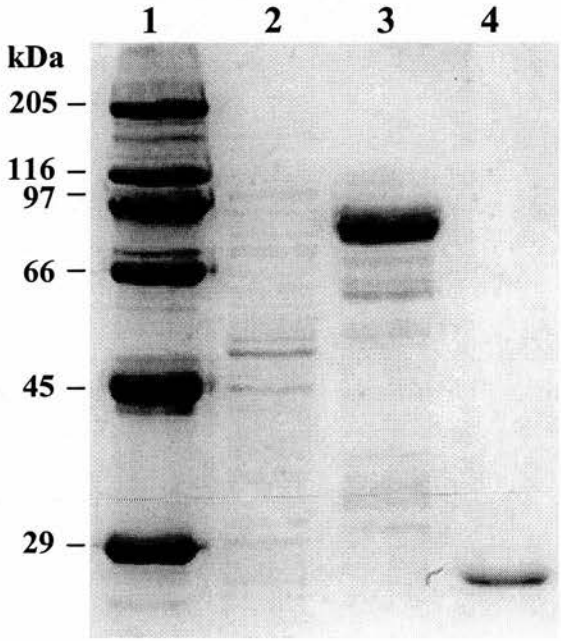
Serum samples	Tamr-1 (no.positive/no.sample)	NC10-Ssp13 (no.positive/no.sample)	Tash-2 (no.positive/no.sample)
Pre-infection	0/12	2/16	1/16
Sporozoites	6/9	4/9	5/9
Low passage macroschizont infected cells	2/3	6/7	3/7
High passage macroschizont infected cells	0/2	1/2	1/2
High passage cells/ sporozoite challenge	2/2	2/2	1/2

5.3.1.1. Tamr-1 antigen

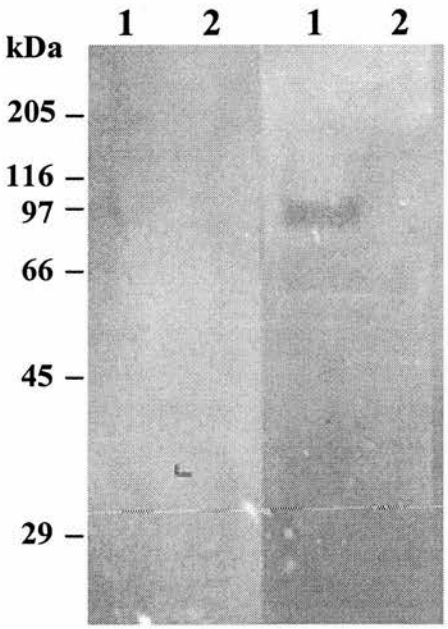
Figure 5.1A shows a Coomassie stained SDS-PAGE gel of purified Tamr-1 (lane 3) and GST (lane 4) recombinant proteins. A distinct band at 116 kDa and several low molecular weight breakdown products were detected with the Tamr-1 fusion protein (Figure 5.1A, lane 3). Pre-infection serum did not react with either the Tamr-1 fusion protein or the GST protein by Western blot analysis (Figure 5.1B).

Figure 5.1. Western blot analysis of purified merozoite rhoptry fusion protein, Tamr-1. A) Coomassie stained 10% SDS-PAGE gel, lane 1: molecular marker, lane 2: Total cell extracts of un-induced bacterial culture; lane 3: Tamr-1 fusion protein, lane 4: GST fusion protein. **B)** Western blot analysis, lane 1 and 3: Tamr-1 fusion protein, lane 2 and 4: GST fusion protein. Lane 1 and 2: probed with pre-infection bovine serum (calf 892A day 0); lane 3 and 4: probed with post-infected bovine serum (calf 892A infected with *T. annulata* Ankara low passage cell line 84 day after infection). The numbers on the left indicate the molecular weights of the marker proteins.

A.



B.



Post-infection bovine serum (day 84) reacted distinctly with the 116 kDa protein band and a range of lower molecular weight proteins between 66 and 100 kDa. Neither pre- nor post-infection bovine serum reacted with the GST protein.

Pre-infection serum samples (n=12) did not react with the Tamr-1 antigen (Table 5.1 Figure 5.2A). Serum samples from two calves immunised with the high passage cell line (*T. annulata* Ankara/Pendik) were negative (Figure 5.2A). Both calves showed positive reaction with the Tamr-1 antigen following challenge (Table 5.1). Four out of 12 serum samples from calves infected either with low passage cell lines or with sporozoites were negative. Antibodies against the Tamr-1 antigen were also detected in the remaining sera.

5.3.1.2. NC10-Ssp13 antigen

Pre-infection sera with two exceptions did not react with the NC10-Ssp13 recombinant antigen (Figure 5.2B; Table 5.1). 13 out of 20 post-infection sera gave positive reactions of varying intensities to the NC10-Ssp13 antigen. Calves immunised with the high passage cell line gave weak bands on Western blots. Following challenge the bands became intense.

5.3.1.3. Tash-2 antigen

All pre-infection serum samples were negative against the recombinant Tash-2 antigen (Figure 5.2C; Table 5.1). 10 out of 20 post-infection sera gave positive signals of different intensities. One of the calves immunised with the high passage cell line gave no reaction after immunisation and challenge.

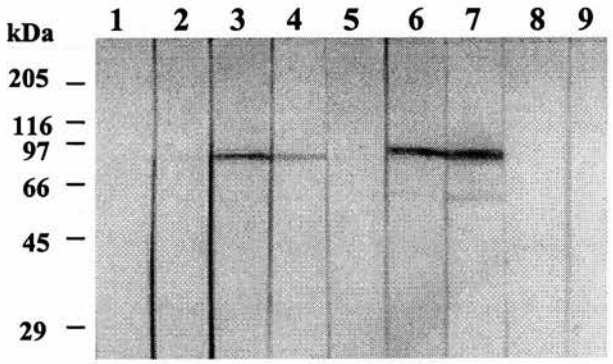
5.3.4. Standardisation and Optimisation of ELISAs

5.3.4.1. Optimisation of reagent dilutions

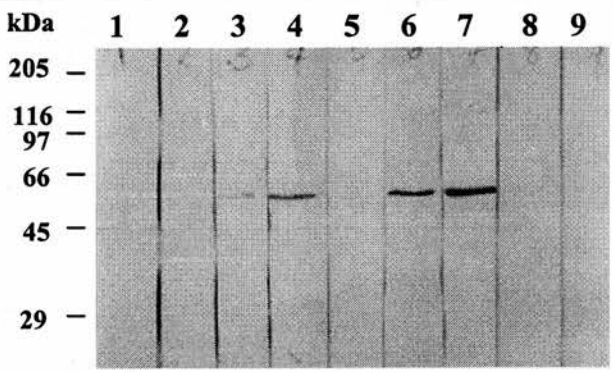
Figures 5.3 to 5.8 show the titrations of the Tamr-1, NC10-Ssp13 and Tash2 recombinant antigens, the reference bovine sera and conjugates. The optimal dilutions determined for the antigens, control sera and conjugates are given in Table 5.2, as are the ratio of positive and negative sera samples (OD_{C++}/OD_{C-}) at the optimal conditions. The same control reference serum samples were used in the IgG Tamr-1, NC10-Ssp13 and Tash-2 ELISAs. The ratio of the positive to negative sera

Figure 5.2. Western blot analysis of three recombinant antigens, Tamr-1, NC10-Ssp13 and Tash-2 using a set of serum samples. A) Tamr-1 fusion protein, **B)** NC10-Ssp13 fusion protein **C)** Tash-2 fusion protein. Lane 1: calf 48C pre-infection, lane 2-3: calf 48C 35 and 112 days after infection with *T. annulata* Ankara sporozoites, lane 4: calf 48C 42 days after challenge with *T. annulata* Gharb sporozoites, lane 5: calf 32C pre-infection, lane 6: calf 32C 35 days after infection with *T. annulata* Ankara low passage cell line, lane 7: calf 32C 42 days after challenge with *T. annulata* Gharb sporozoites, lane 8: calf 891A pre-infection, lane 9: calf 891A 28 days after immunisation with *T. annulata* Ankara/Pendik low passage cell line. 100 ng of protein was loaded into each lane. Molecular size markers (kDa) are indicated on the left.

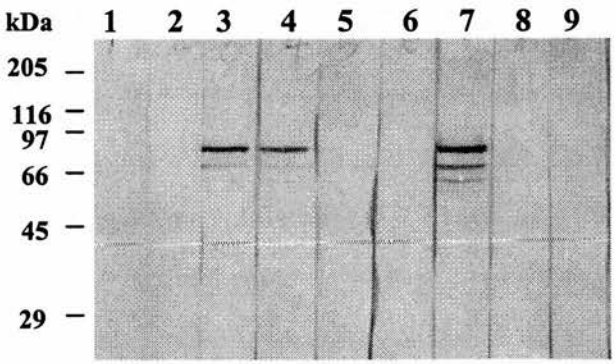
A.


kDa
1 2 3 4 5 6 7 8 9
205
116
97
66
45
29

B.


kDa
1 2 3 4 5 6 7 8 9
205
116
97
66
45
29

C.


kDa
1 2 3 4 5 6 7 8 9
205
116
97
66
45
29

obtained with the IgG Tamr-1 ELISA was greater than those obtained for both IgG NC10-Ssp13 and Tash-2 ELISAs (Table 5.2). The results showed that with increasing serum dilutions, the OD readings decreased more rapidly in the Tamr-1 ELISA than in the NC10-Ssp13 and Tash-2 ELISAs (Figure 5.3, 5.4, 5.5).

IgG NC10-Ssp13 ELISA gave higher background binding OD levels with negative serum samples compared to the Tamr-1 and Tash-2 antigens (Figure 5.4). Because of time constraints, different dilutions of blocking agents (Skimmed milk, Tween 20 etc.) could not be tested in order to reduce the non-specific background binding.

The high positive control serum (C++) used at 1:500 and 1:100 for the Tamr-1 and NC10-Ssp13 respectively gave very high OD values in the Tash-2 ELISA (Figure 5.5) at 1:400 dilution. Similar results were obtained with other C++ sera (data not shown).

The control serum samples (C++ and C+) used in IgG ELISAs were not suitable for the IgM ELISAs. High positive control serum (C++) gave a very high OD value (around 2.5 OD) at optimal conditions (data not shown). Therefore, new control serum samples with acceptable positive/negative OD ratios were chosen (Table 5.2).

The Tamr-1 antigen gave higher background binding OD values in IgM ELISA in comparison the IgG ELISA. With increasing serum dilutions, the OD reading decreased more rapidly in the Tamr-1 ELISA than in the NC10-Ssp13 and Tash-2 ELISAs (Figure 5.6, 5.7, 5.8).

High antigen dilutions were required for both NC10-Ssp13 and Tash-2 antigens in order to detect *T. annulata* specific IgM antibodies in C+ control serum sample (Figures 5.7A, 5.8A). When low antigen concentrations were used lower background binding OD levels were obtained. The ratios of the positive to negative sera obtained with the IgM NC10-Ssp13 and Tash-2 ELISAs were greater than that obtained for the Tamr-1 ELISA (Table 5.2). The antibody titres obtained in C+ with IgM NC10-Ssp13 and Tash-2 ELISAs control serum sample gave low OD readings such that C+ and C- sera could not be distinguished from each other unequivocally (Figures 5.7, 5.8).

Table 5.2. Optimal reagent dilutions of standardised *Theileria annulata* antibody ELISAs for testing bovine sera and the ratio of positive to negative control optical densities at the optimal reagent dilutions.

A) IgG isotype	Tamr-1	NC10-Ssp13	Tash-2
Recombinant antigen	1:3,200 (1.0 μg/ml)	1:100 (1.6 μg/ml)	1:1,600 (0.08 μg/ml)
Serum	1:500	1:100	1:400**
Conjugate (HRPO)	1:10,000	1:5,000	1:8,000
Ratio of positive to negative sera	15.5	4.0	9.5
B) IgM isotype	Tamr-1	NC10-Ssp13	Tash-2
Recombinant antigen	1:3,200 (1.0 μg/ml)	1:3,200 (0.05 μg/ml)	1:3,200 (0.04 μg/ml)
Serum	1:400	1:200	1:200
IgM	1:20,000	1:10,000	1:10,000
ExtrAvidin*	1:1,000	1:1,000	1:1,000
Ratio of positive to negative sera	4.1	13.1	15.4

*Dilution recommended by supplier

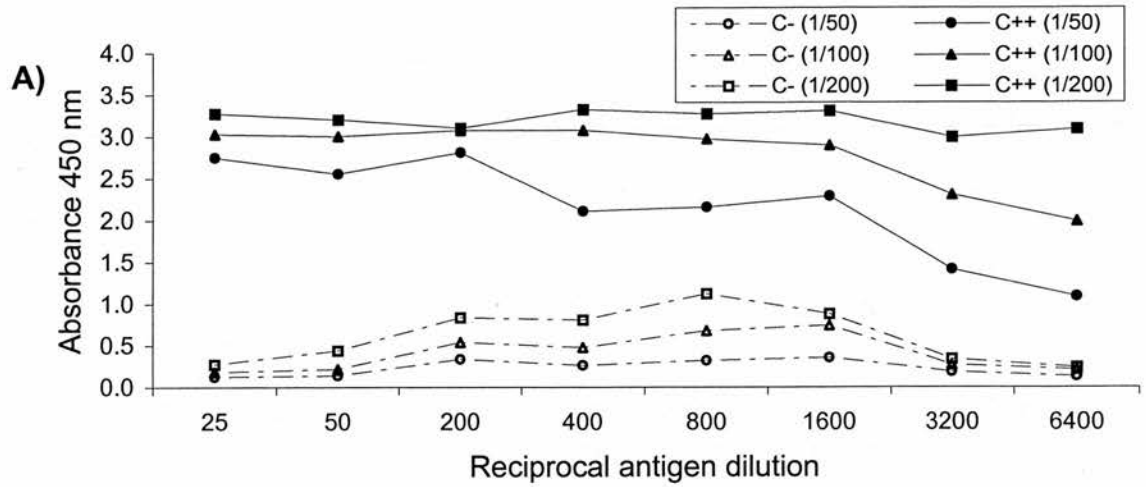
**C++ control serum used at 1/800 dilution due to high OD values.

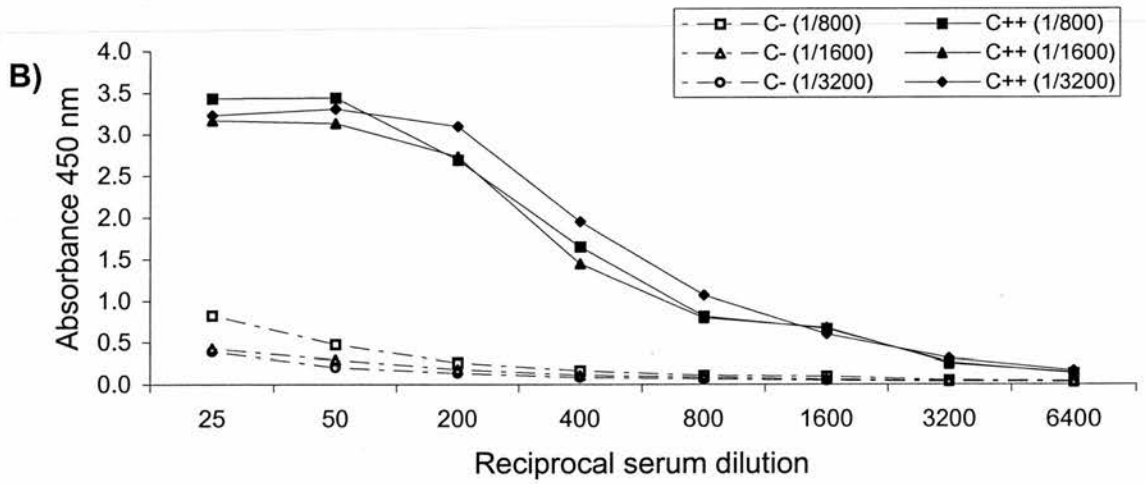
Figure 5.3. Titration of the antigen, control serum samples and conjugates in the standardisation of IgG Tamr-1 ELISA.

A) Tamr-1 antigen dilutions (1.4 mg/ml-0.54 μg/ml, i.e. 1:25-1:6,400) using control serum samples at the dilution as shown in brackets (1:50-1:200) and a 1:5,000 conjugate dilution.

B) Titration of control serum samples (C-, C++) using dilutions of Tamr-1 (1:800-1:3,200) and conjugate (1:5,000).

C) Titration of the conjugate using the optimal dilution of Tamr-1 (1:1,600, i.e. 1.1 μg/ml) and control serum samples (C-, C++) (1:400-1:600).


A)
C- (1/50)
C++ (1/50)
C- (1/100)
C++ (1/100)
C- (1/200)
C++ (1/200)
Absorbance 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
200
400
800
1600
3200
6400
Reciprocal antigen dilution


B)
C- (1/800)
C++ (1/800)
C- (1/1600)
C++ (1/1600)
C- (1/3200)
C++ (1/3200)
Absorbance 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
200
400
800
1600
3200
6400
Reciprocal serum dilution

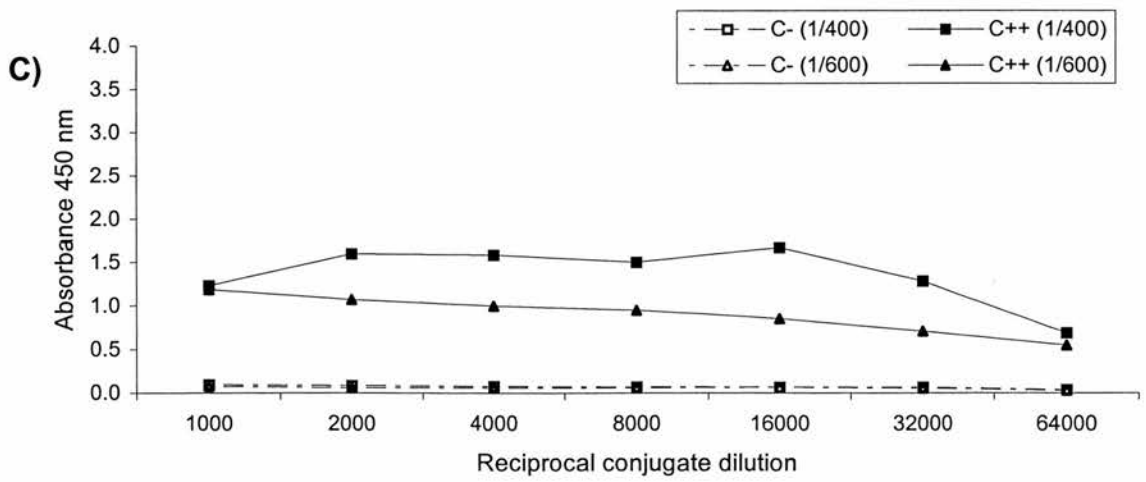

C)
C- (1/400)
C++ (1/400)
C- (1/600)
C++ (1/600)
Absorbance 450 nm
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
1000
2000
4000
8000
16000
32000
64000
Reciprocal conjugate dilution

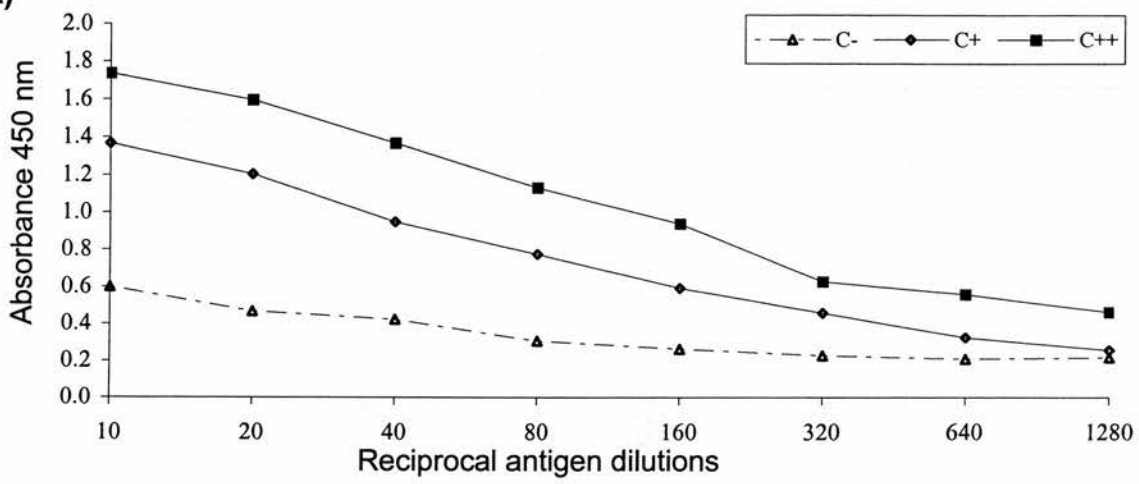
Figure 5.4. Titration of the antigen, control serum samples and conjugates in the standardisation of IgG NC10-Ssp13 ELISA.

A) NC10-Ssp13 antigen dilutions (16 μg/ml-0.13 μg/ml, i.e. 1:10-1:1280) using control serum samples (C-, C+, C++) and conjugate at dilutions 1:50 and 1:5,000 respectively.

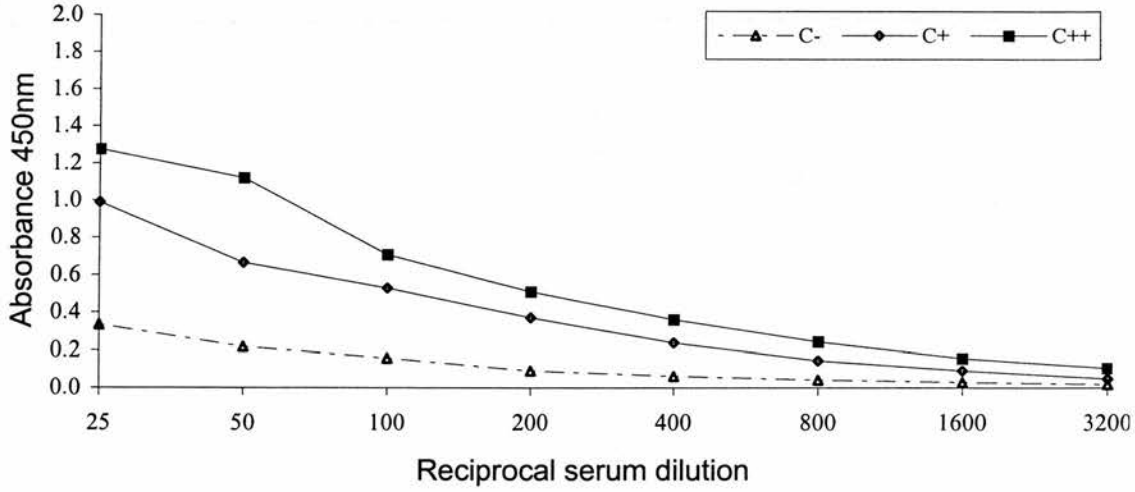
B) Titration of control serum samples (C-, C+, C++) using the optimal dilution of NC10-Ssp13 (1.6 μg/ml, i.e. 1:100) and conjugate dilution at 1:5,000.

C) Titration of the conjugate using optimal dilution of NC10-Ssp13 (1.6 μg/ml) and control serum samples (C-, C+, C++) at dilution 1:100.

A)


C-
C+
C++
Absorbance 450 nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10
20
40
80
160
320
640
1280
Reciprocal antigen dilutions

B)


C-
C+
C++
Absorbance 450nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
25
50
100
200
400
800
1600
3200
Reciprocal serum dilution

C)

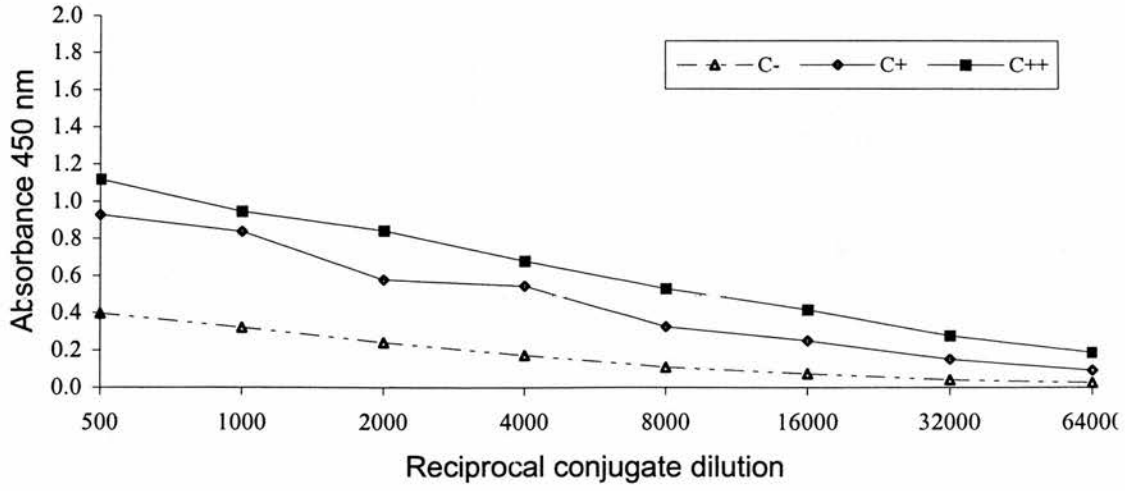
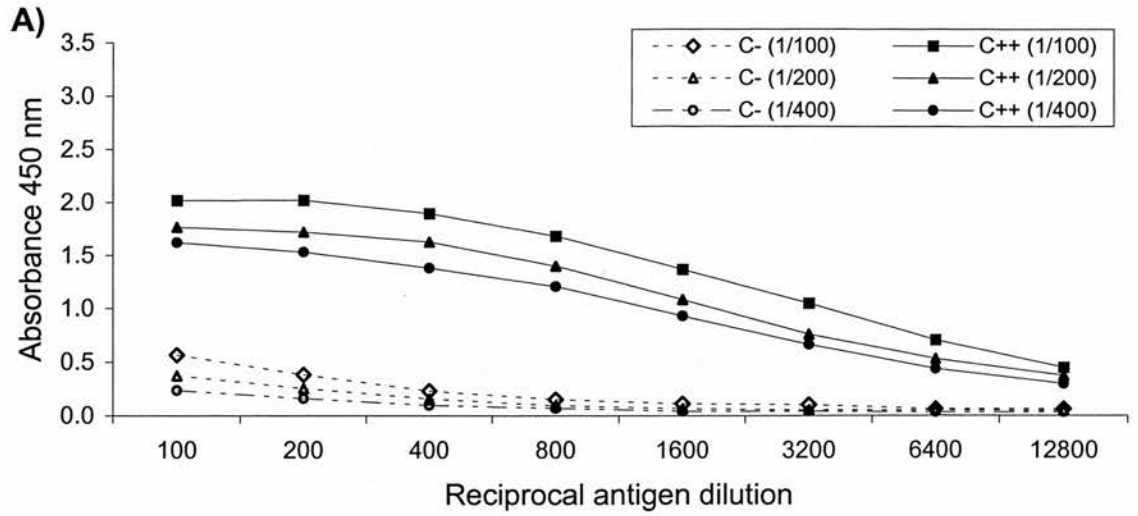

C-
C+
C++
Absorbance 450 nm
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
500
1000
2000
4000
8000
16000
32000
Reciprocal conjugate dilution

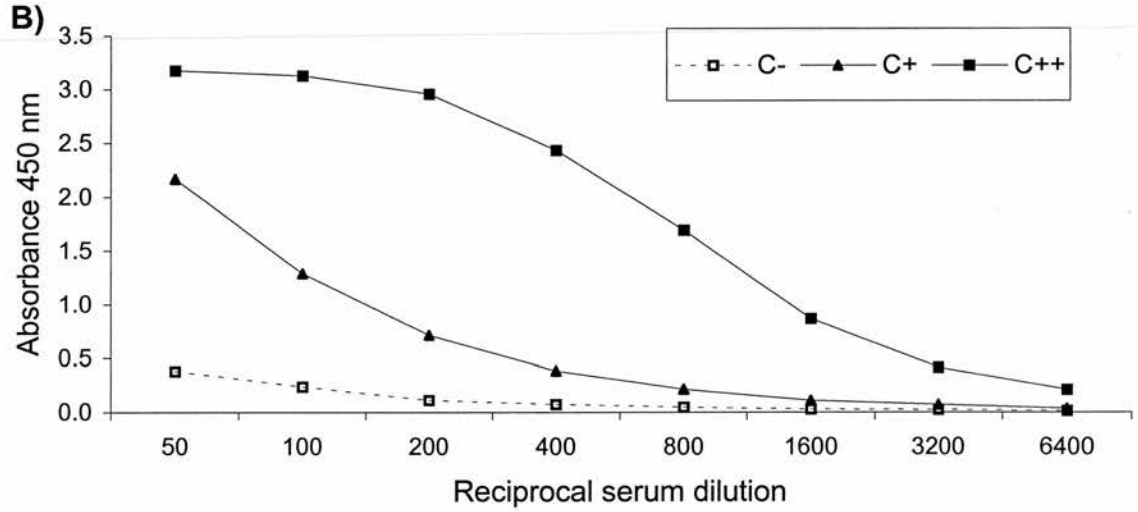
Figure 5.5. Titration of the antigen, control serum samples and conjugates in the standardisation of IgG Tash-2 ELISA.

A) Tash-2 antigen dilutions (1.3 μg/ml-0.01 μg/ml, i.e. 1:100-1:12,800) using control serum samples (C-, C++) at 1:100-1:400 dilutions and conjugate at 1:5,000 dilution.

B) Titration of control serum samples (C-, C+, C++) using the optimal dilution of Tash-2 (80 ng/ml, i.e. 1:1,600) and conjugate dilution at 1:5,000.

C) Titration of the conjugate using the optimal dilution of Tash-2 (80 ng/ml) and control serum samples (C-, C+, C++) at 1:400 and 1:800 dilutions as indicated in brackets.


A)
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C- (1/100)
C- (1/200)
C- (1/400)
C++ (1/100)
C++ (1/200)
C++ (1/400)
100
200
400
800
1600
3200
6400
12800
Reciprocal antigen dilution


B)
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C-
C+
C++
50
100
200
400
800
1600
3200
6400
Reciprocal serum dilution

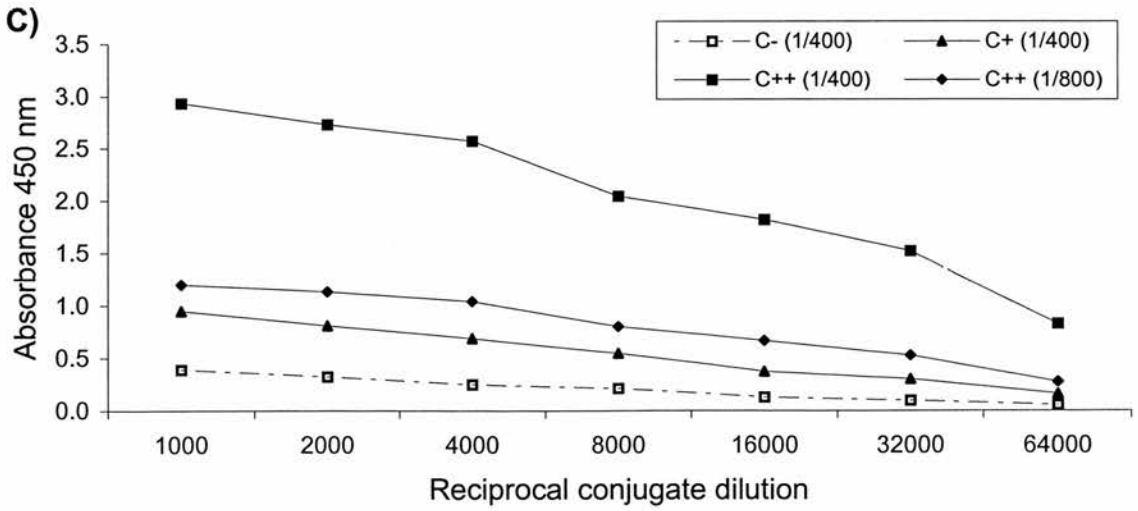
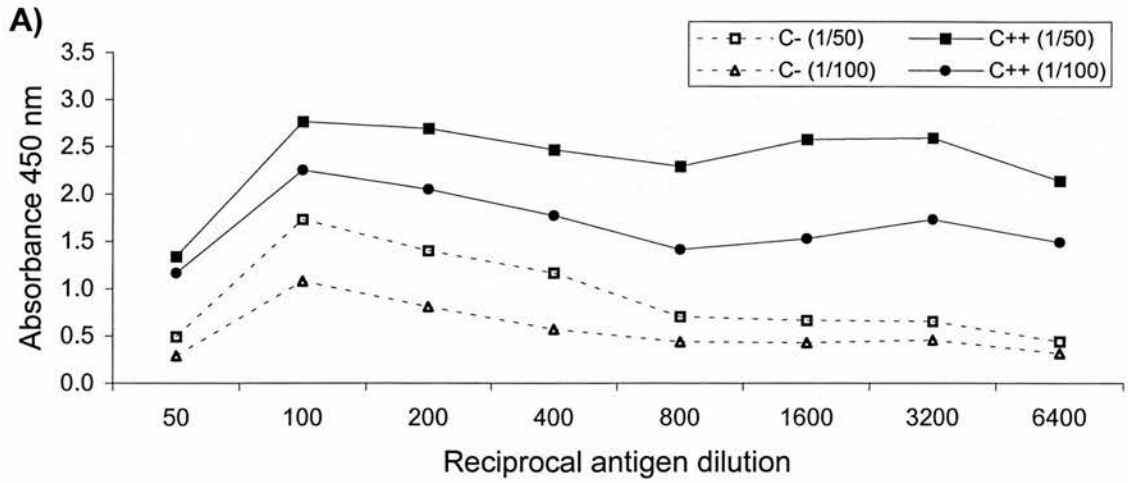

C)
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
C- (1/400)
C+ (1/400)
C++ (1/400)
C++ (1/800)
1000
2000
4000
8000
16000
32000
64000
Reciprocal conjugate dilution

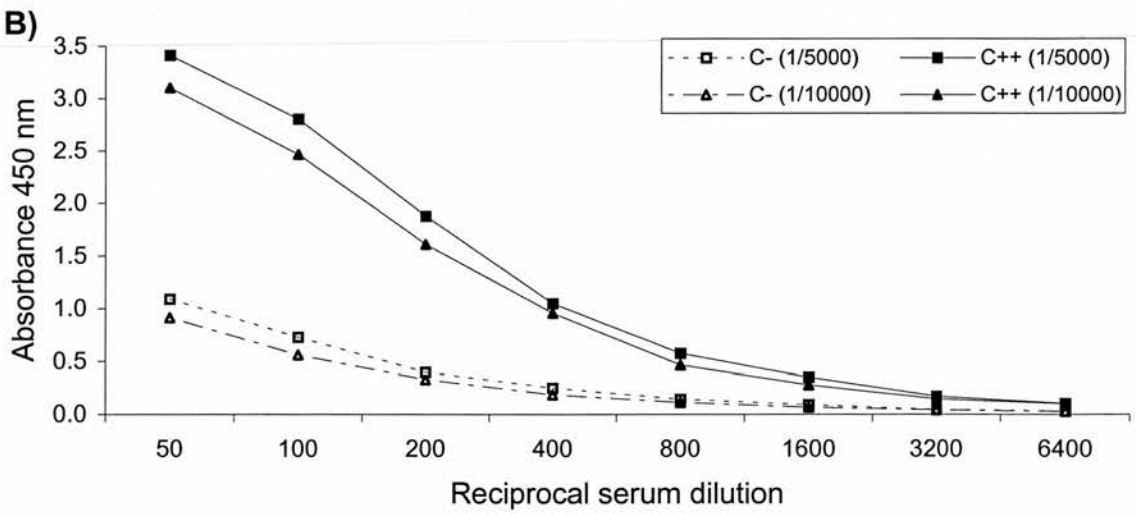
Figure 5.6. Titration of the antigen, control serum samples and conjugates in the standardisation of IgM Tamr-1 ELISA.

A) Tamr-1 antigen dilutions (1.4 mg/ml-0.54 μg/ml, i.e. 1:25-1:6400) using control serum samples (C-, C++) at 1:50 and 1:100 dilutions. The dilutions of IgM monoclonal antibody and ExtrAvidin conjugate were 1:10,000 and a 1:1,000, respectively.

B) Titration of control serum samples (C-, C++) using the optimal dilution of Tamr-1 (1.0 μg/ml, i.e. 1:1,600). The dilutions of IgM monoclonal antibody and ExtrAvidin conjugate were 1:10,000 and a 1:1,000, respectively.

C) Titration of the conjugate using the optimal dilutions of Tamr-1 (1.0 μg/ml), control serum samples (C-, C++) at 1:200-1:400 and ExtrAvidin conjugate at 1:1,000.


A)
C- (1/50)
C++ (1/50)
C- (1/100)
C++ (1/100)
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50
100
200
400
800
1600
3200
6400
Reciprocal antigen dilution


B)
C- (1/5000)
C++ (1/5000)
C- (1/10000)
C++ (1/10000)
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50
100
200
400
800
1600
3200
6400
Reciprocal serum dilution

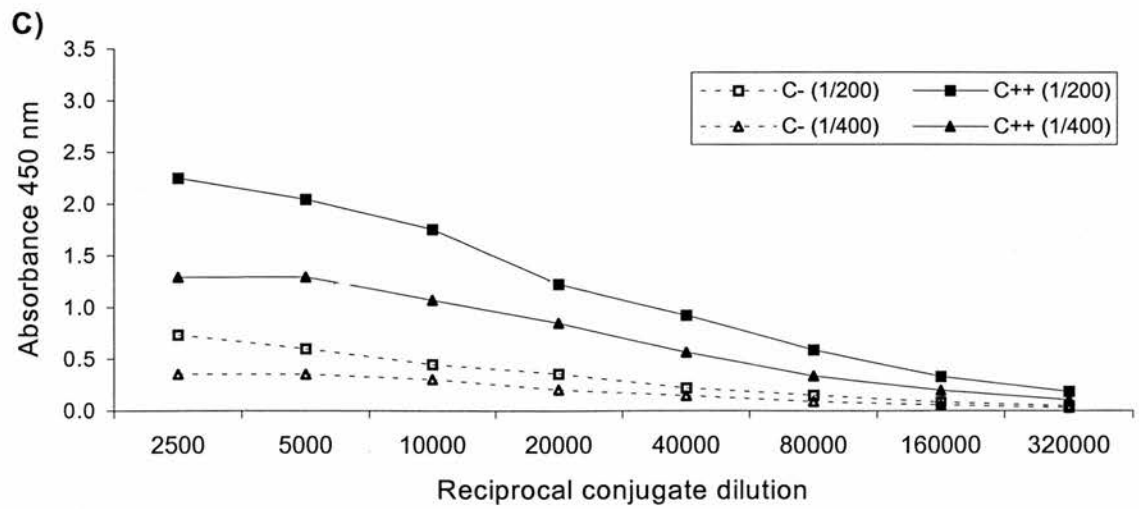
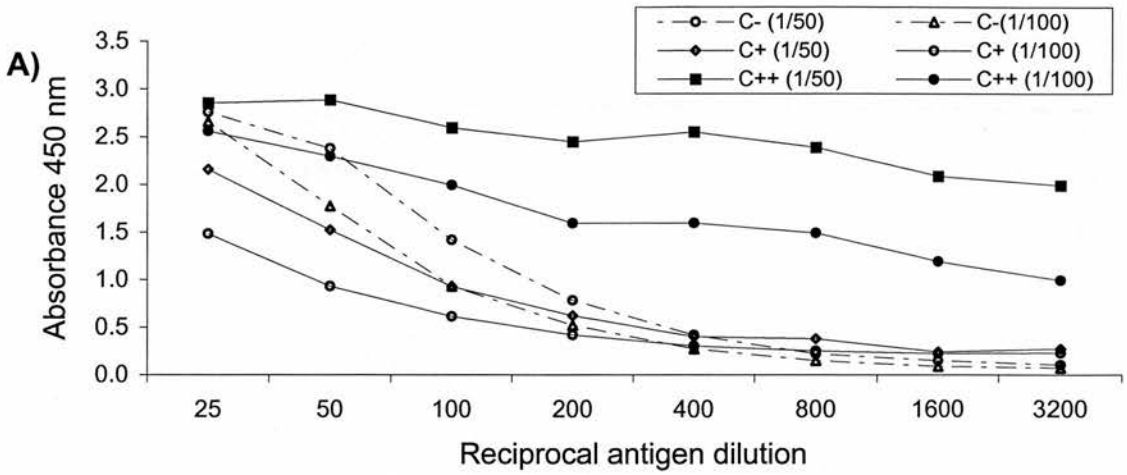

C)
C- (1/200)
C++ (1/200)
C- (1/400)
C++ (1/400)
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2500
5000
10000
20000
40000
80000
160000
320000
Reciprocal conjugate dilution

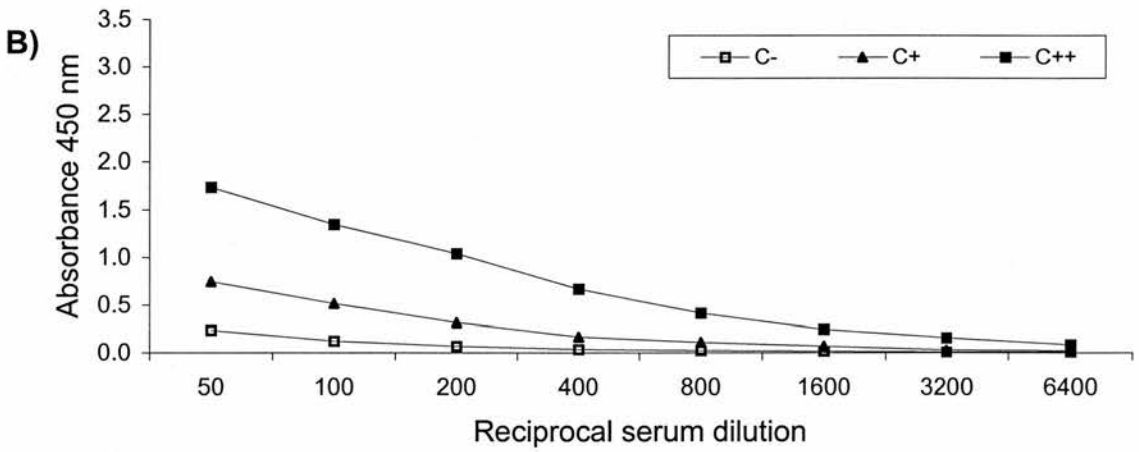
Figure 5.7. Titration of the antigen, control serum samples and conjugates in the standardisation of IgM NC10-Ssp13 ELISA.

A) NC10-Ssp13 antigen dilutions (6.4 μg/ml-50 ng/ml, i.e. 1:25-1:3,200) using control serum samples (C-, C+, C++) at 1:50 and 1:100 dilutions. The dilutions of IgM monoclonal antibody and ExtrAvidin conjugate were 1:10,000 and a 1:1,000, respectively.

B) Titration of control serum samples (C-, C+, C++) using the optimal dilution of NC10-Ssp13 (50 ng/ml, i.e. 1:3,200). The dilutions of IgM monoclonal antibody and ExtrAvidin conjugate were 1:10,000 and a 1:1,000, respectively.

C) Titration of the conjugate using optimal dilutions of NC10-Ssp13 (50 ng/ml), control serum samples (C-, C+, C++) at 1:200 and ExtrAvidin conjugate at 1:1,000.


A)
C- (1/50)
C-(1/100)
C+ (1/50)
C+ (1/100)
C++ (1/50)
C++ (1/100)
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
100
200
400
800
1600
3200
Reciprocal antigen dilution


B)
C-
C+
C++
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50
100
200
400
800
1600
3200
6400
Reciprocal serum dilution

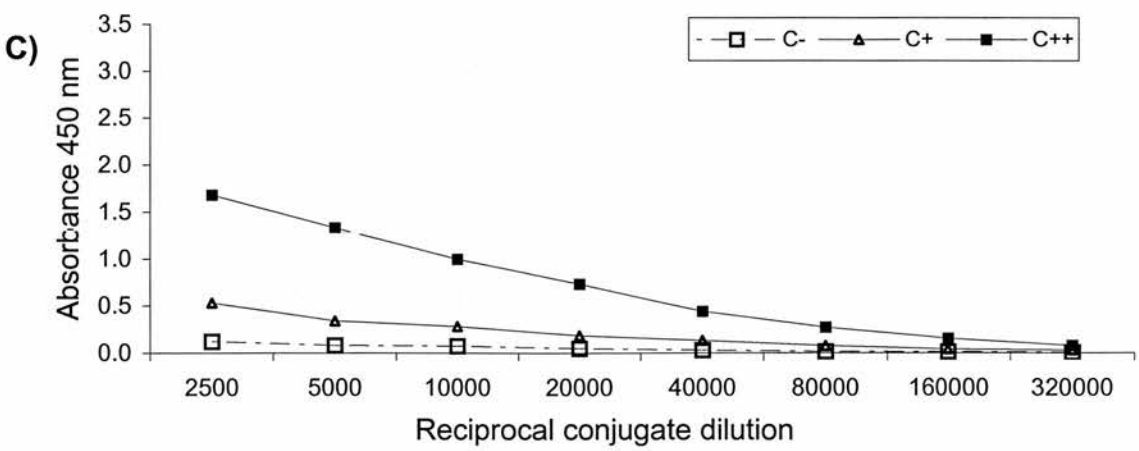
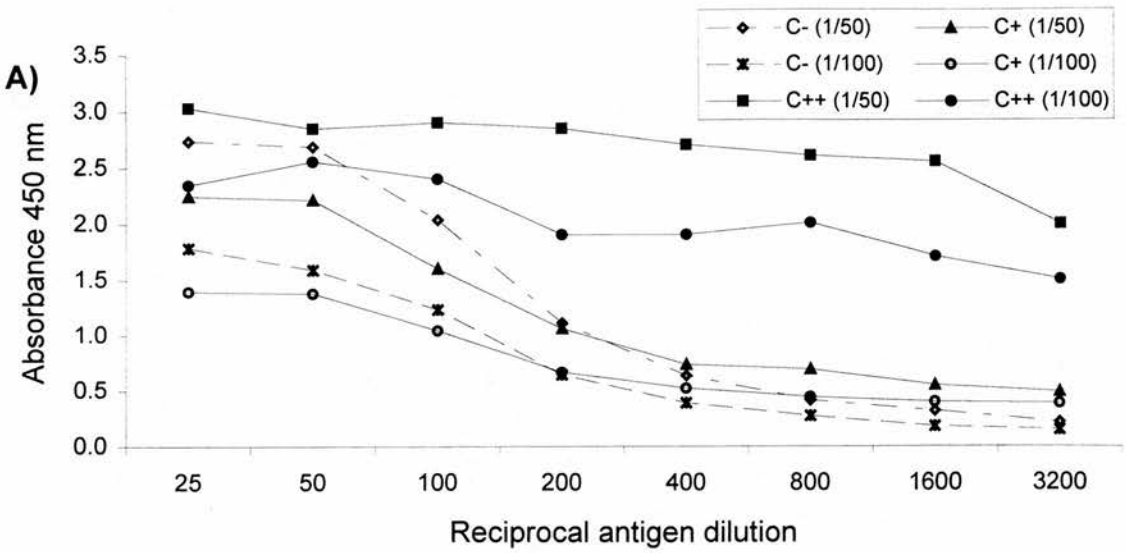

C)
C-
C+
C++
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2500
5000
10000
20000
40000
80000
160000
320000
Reciprocal conjugate dilution

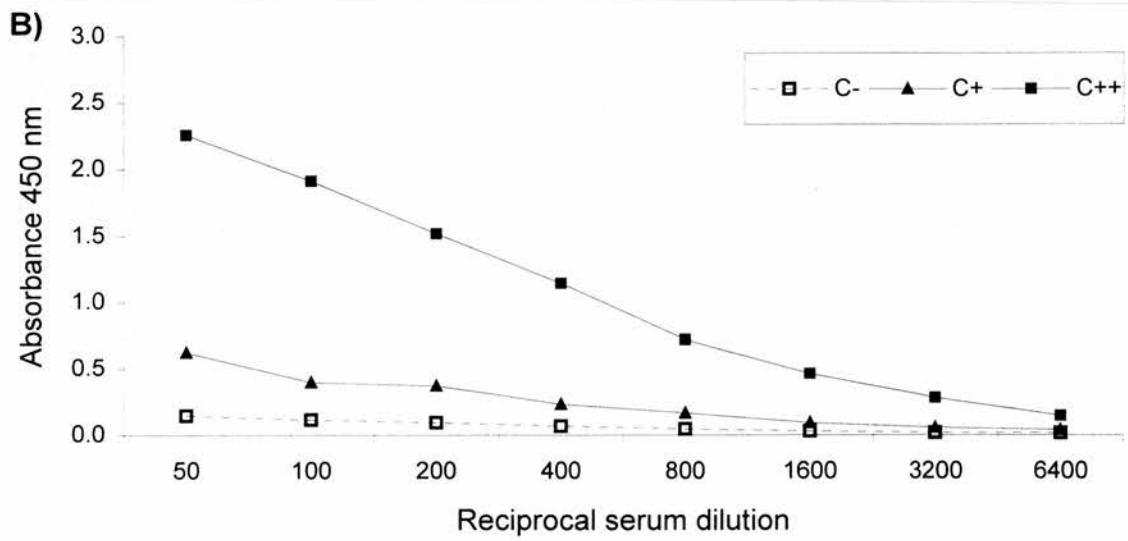
Figure 5.8. Titration of the antigen, control serum samples and conjugates in the standardisation of IgM Tash-2 ELISA.

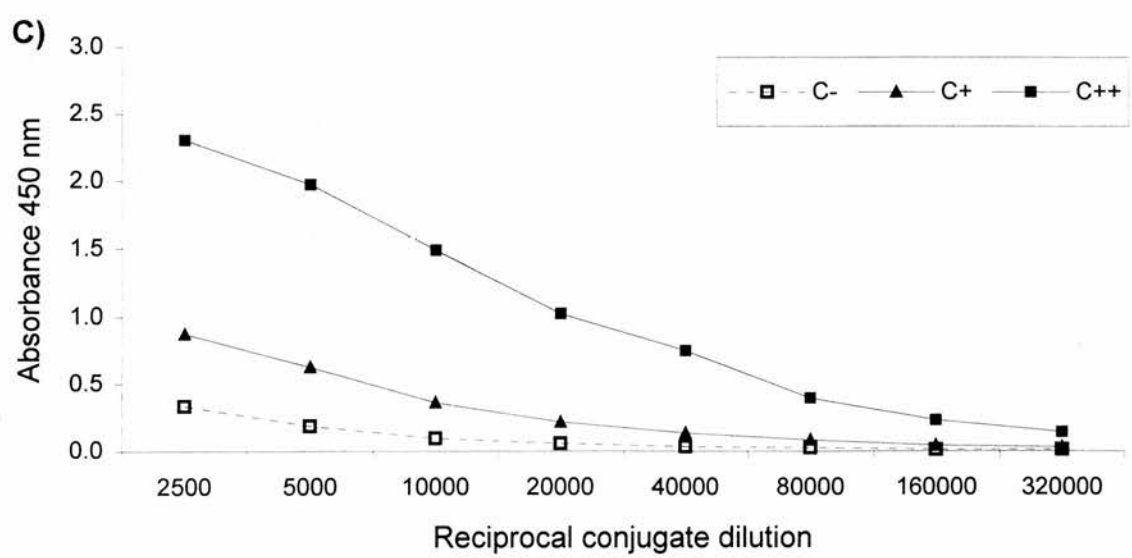
A) Tash-2 antigen dilutions (5.2 μg/ml- 40 ng/ml, i.e. 1:25-1:3,200) using control serum samples (C-, C+, C++) at 1:50 and 1:100 dilutions. The dilutions of IgM monoclonal antibody and ExtrAvidin conjugate were 1:10,000 and a 1:1,000, respectively.

B) Titration of control serum samples (C-, C+, C++) using optimal dilution of Tash-2 (40 ng/ml, i.e. 1:3,200). The dilutions of IgM monoclonal antibody and ExtrAvidin conjugate were 1:10,000 and a 1:1,000, respectively.

C) Titration of the conjugate using optimal dilutions of Tash-2 (40 ng/ml), control serum samples (C-, C+, C++) at 1:200 and ExtrAvidin conjugate at 1:1,000 dilution.


A)
C- (1/50)
C+ (1/50)
C- (1/100)
C+ (1/100)
C++ (1/50)
C++ (1/100)
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
25
50
100
200
400
800
1600
3200
Reciprocal antigen dilution


B)
C-
C+
C++
Absorbance 450 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
50
100
200
400
800
1600
3200
6400
Reciprocal serum dilution


C)
C-
C+
C++
Absorbance 450 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
2500
5000
10000
20000
40000
80000
160000
320000
Reciprocal conjugate dilution

5.3.4.2. Establishment of upper and lower control limits

Upper and lower control limits (LCL and UCL) established for the IgG Tamr-1 ELISA are given in Table 5.3. PP values were calculated using median OD_{C++} value of 1.264. In the IgG Tamr-1 ELISA, there was no significant difference between inner and outer replicate wells in the repeated quality control plates ($p<0.001$). Therefore, results of all wells were accepted as consistent.

Table 5.3. Upper (UCL) and lower (LCL) control limits for IgG Tamr-1 ELISA given as optical densities. Percent positivity (PP) values are given in brackets.

Control	UCL	LCL
C++	1.536 (121.5%)	1.045 (82.7%)
C+	0.499 (39.5%)	0.341 (27.0%)
C-	0.072 (5.7%)	0.050 (4.0%)
Cc	0.016 (1.3%)	0.006 (0.5%)

5.3.4.3. Sensitivity and specificity of the Tamr-1 antigen.

The frequency distribution of PP values of IgG Tamr-1 ELISA from the negative (n=188) and positive (n=209) sera is given in Figure 5.9. Figure 5.10A and B shows the results of TG-ROC analysis indicating the relationship between cut-off values and the sensitivity and specificity of the IgG Tamr-1 ELISA.

The mean of duplicate sample PP values was arranged as positive or negative, using cut-off values of 14 PP and 18 PP on the basis of 95% and 99% specificity of the test. At a cut-off value of 14 PP, sensitivity and specificity were 78.5% [73.0-84.1 with a 95% confidence interval, (CI)] and 95.2% (92.0-98.3, 95% CI), respectively. At a cut-off value of 18 PP, sensitivity and specificity were 72.7% (66.7-78.7, 95% CI) and 98.4% (96.7-100, 95% CI), respectively (Table 5.4).

Serum samples from animals immunised with high passage, attenuated cell lines were used to assess the sensitivity of the Tamr-1 IgG ELISA. The percent positivity of most of these serum samples was under the chosen cut-off points (14 PP and 18 PP) (Table 5.4). Although the number of sera tested from high passage cell

Figure 5.9. Distribution of percentage positive (PP) data for sera from *T. annulata* infected (positive, n=209) and uninfected (negative, n= 188) cattle.

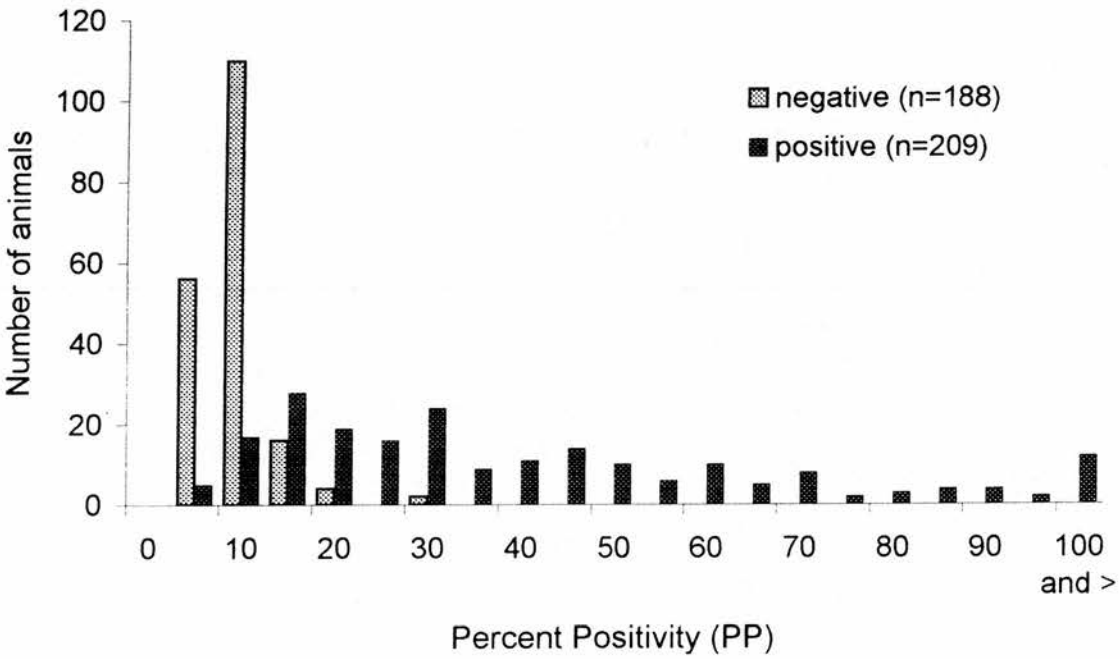
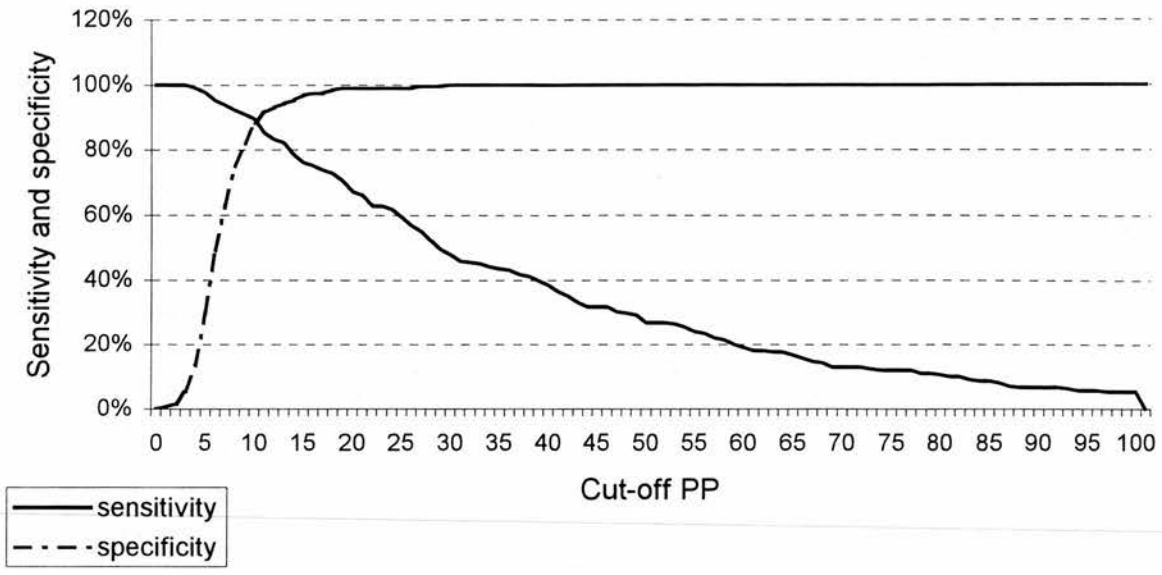

120
100
80
60
40
20
0
Number of animals
negative (n=188)
positive (n=209)
0
10
20
30
40
50
60
70
80
90
100
and >
Percent Positivity (PP)

Figure 5.10. TG-ROC analysis of the IgG Tamr-1 ELISA, indicating the relationship between cut-off values and the sensitivity and specificity of the IgG Tamr-1 ELISA. A) Serum samples obtained from animals infected either with sporozoites or with low and high passage cell lines were used to determine sensitivity and specificity (positive, n=209; negative, n= 188). **B)** Serum samples obtained from animals infected either with sporozoites or with low passage cell lines were used to determine sensitivity and specificity (positive, n=183; negative, n= 188).

A)


120%
100%
80%
60%
40%
20%
0%
Sensitivity and specificity
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Cut-off PP
sensitivity
specificity

B)

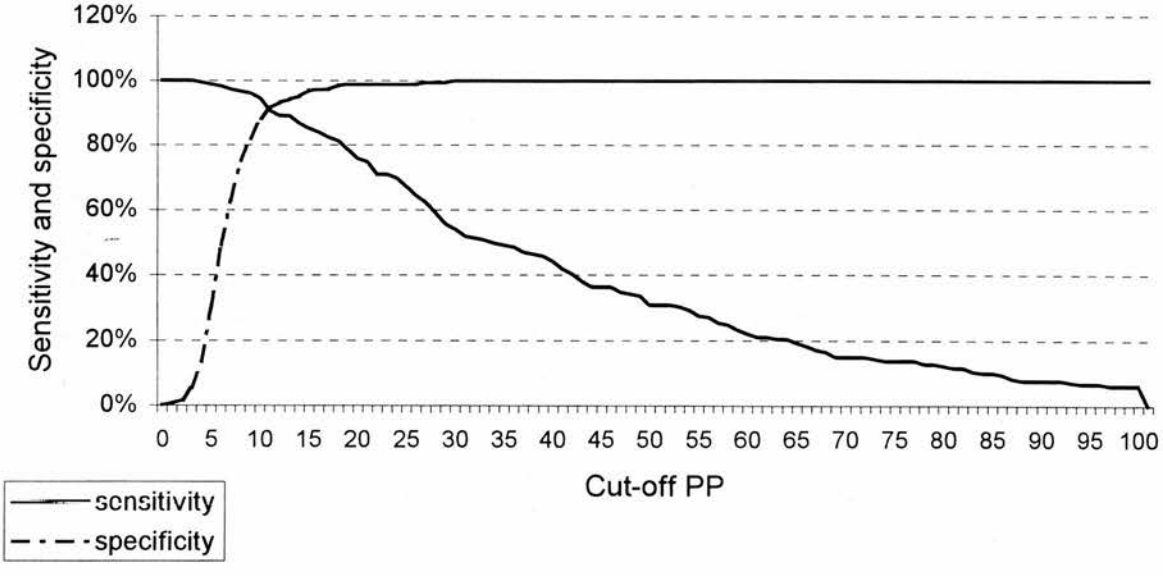

120%
100%
80%
60%
40%
20%
0%
Sensitivity and specificity
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100
Cut-off PP
sensitivity
specificity

Table 5.4. Estimates of diagnostic sensitivity (%) with associated 95% confidence intervals (CI) in brackets for IgG Tamr-1 ELISA calculated for animals infected with *T. annulata* sporozoites or immunised with either low passage or high passage cell lines. Groups with different letters are statistically significant from one another.

Cut-off	Sporozoite[A] (n: 86)	Low passage[A] (n: 97)	High passage[B] (n: 26)	All (n: 209)
14 PP	83.7 (75.9-91.5)	88.7 (82.4-95.0)	23.1 (6.8-39.4)	78.5 (73.0-84.1)
18 PP	77.9 (69.1-86.7)	83.5 (76.1-90.9)	15.4 (1.5-29.3)	72.7 (66.7-78.7)

line immunised animals was small, exclusion of serum samples obtained from animals immunised with the high passage cell lines improved the sensitivity of the test. The sensitivity of the test differed slightly between serum samples from animals immunised with the low passage cell lines and those from animals infected with sporozoites. However, results obtained from sporozoite infected animals were not significantly different from macroschizont infected animals at both given cut-off values of 14 PP ($p = 0.332$) and 18 PP ($p = 0.337$). Some serum samples from animals immunised with cell lines *T. annulata* Tova and *T. annulata* Ode, which do not have the ability to produce piroplasms *in vivo,* gave positive antibody response to the Tamr-1 antigen (data not shown).

If the test specificity was reduced to 90% at 11 PP cut-off value, the sensitivity of the IgG Tamr-1 ELISA was 85.2% (80.4-90.0, 95% CI) including all sera (Figure 5.10A). When serum samples from animals immunised with the high passage cell lines were excluded the sensitivity of the test at 11 PP cut-off value was 90.7% (86.5-94.9, 95% CI) (Figure 5.10B), which is not significantly different from the sensitivity obtained with the inclusion of the high passage cell line.

A substantial agreement between the piroplasm antigen IFAT and the IgG Tamr-1 ELISA was found when all serum samples were included (*kappa*= 0.73 at 14 PP and 0.7 at 18 PP). The agreement between the two tests was also substantial at each cut-off value using serum samples from animals infected with sporozoites (*kappa*= 0.8 at both 14PP and 18 PP). Almost perfect agreement between the two tests was observed with samples from animals immunised with the low passage cell line (*kappa*= 0.84 at both 14PP and 18 PP).

The results of the cross-reactivity of the IgG Tamr-1 ELISA with serum samples from experimentally infected calves (or field samples from Sicily) are given in Table 5.5. Of 15 serum samples from *T. parva*-infected calves, eight were positive at 14 PP and five at 18 PP. Of the two *T. buffeli* positive samples from Australia, neither was positive at both cut-off values. However, four out of ten *T. buffeli* positive serum samples from Sicily were positive at the cut-off value of 18 PP. One animal infected with *B. bovis* and one infected with *Trypanosoma evansi* were positive at the cut-off value of 14 PP.

Table 5.5. The results of the cross-reactivity of the IgG Tamr-1 ELISA with serum samples from experimentally infected calves (or field samples from Sicily).

Infection with	**No. Positive / No. Sample**	
	14 PP cut-off	**18 PP cut-off**
T. parva	8/15	5/15
T. buffeli	4/12	4/12
T. sergenti	0/2	0/2
Babesia bovis	1/12	0/12
B. bigemina	0/9	0/9
Anaplasma marginale	0/8	0/8
Trypanosoma evansi	1/2	0/2
Ostertagia ostertagi	0/3	0/3

5.3.4.4 Blind Test

Table 5.6 shows the results of the blind test performed at the CTVM using the IgG Tamr-1 ELISA in correlation with the results of IFAT using the piroplasm antigen. At cut-off points of 14 PP and 18 PP, 91.7% (86.2-97.2, 95% CI) and 95.8% (91.8-99.8, 95% CI) of animals were true negative, respectively. The proportion of IFA positive animals were 90.3% (85.5-95.1, 95% CI) and 75% (67.9-82.1, 95% CI) at cut-off points of 14 PP and 18 PP, respectively. Thirteen animals that were infected with various cell lines, *viz. T. annulata* Tova, which does not produce piroplasms, and *T. annulata* Ode, which is an attenuated cell line, were tested. Two out of three *T. annulata* Tova serum samples and five out of six *T. annulata* Ode serum samples were above 18 PP. Since the standardisation tests had expected counts less than five at 18 PP cut-off value, Chi-square test could not be used. Instead, the Fisher's exact test was used (Instat statistic). At 18 PP cut-off value, there were significant differences in the sensitivity of the test from the values determined in the standardisation and optimisation section 5.3.4.3 ($p < 0.05$). The specificity of the test was not significantly different at 14 PP cut-off value. There was no significant difference of both groups of sera. Serum samples from animals infected with *T. buffeli, B. bigemina* and *B. bovis* were negative. Two out of six samples obtained from animals infected with *T. parva* were positive, one at 14 PP and another at 18 PP.

Table 5.6. Results of the IgG Tamr-1 ELISA and the piroplasm IFAT using serum samples in blind test.

ELISA		No. of samples with IFAT		Totals
		Positive	Negative	
Cut-off 14 PP	Positive	130	8	138
	Negative	14	88	102
	Total	144	96	240
Cut-off 18 PP	Positive	108	4	112
	Negative	36	92	128
	Totals	144	96	240

5.4. DISCUSSION

The current study was designed to assess the performance of three recombinant antigens, Tamr-1, NC10-Ssp13 and Tash-2, in ELISAs with the main objective of distinguishing animals vaccinated against tropical theileriosis from those that are naturally infected with *T. annulata*. Three recombinant antigens expressed in different life cycle stages of the parasite, Tamr-1 (merozoite/piroplasm), NC10-Ssp13 (macroschizont) and Tash-2 (macroschizont /piroplasm) were shown to be antigenic in Western blot analysis with the majority of sera obtained from animals either infected with sporozoites or immunised with low passage cell lines of various stocks of *T. annulata*. Consequently, ELISAs performed with these three recombinant antigen could be used as diagnostic tools for *T. annulata* infection in cattle. Since the primary aim of the study was the development of an ELISA that can be used to detect only naturally infected animals but not vaccinated ones, detailed studies were carried out with the IgG Tamr-1 ELISA. Only preliminary standardisation was performed for the NC10-Ssp13 and Tash-2 ELISAs because of time constraints.

The indirect ELISA format was selected to detect IgG and IgM antibodies against *T. annulata* infection. The same basic protocol was used for each IgG and IgM ELISA in terms of the incubation times and substrate. However, reagent dilutions were optimised separately for each ELISA by chequerboard titration. Plate-to-plate and day-to day variations are major sources of experimental error in ELISA assays. In order to minimise these variations, large batches of reagents and plates were obtained and stored at recommended conditions. To obtain upper and lower control limits, 90^{th} and 10^{th} percentiles were considered to be more appropriate because the data from repeated assays did not have a normal distribution (Davison *et al.*, 1999).

Pre-infection serum samples tested in both IgG and IgM NC10-Ssp13 ELISAs gave relatively high background binding. It is necessary to optimise several parameters such as plate type, the content and pH of the buffer, incubation temperature and length, as suggested previously (Kemeny, 1992; Venkatesan and Wakelin, 1993). pH of the buffer probably influences the charge property of the solid phase leading to non-specific binding of serum immunoglobulins, especially IgM

(Kuen *et al.*, 1993). High background binding observed with the NC10-Ssp13 ELISA could also be due to the recombinant antigen itself. The fact that the NC10-Ssp13 recombinant protein used in the current study contains part of the repeat region of the protein (see Chapter 4) may lead to cross-reactions with other parasites or pathogens with similar epitopes.

The diagnostic sensitivity and specificity of the IgG Tamr-1 ELISA was determined using tests performed with 209 positive sera from experimentally infected or immunised cattle and 188 negative sera from British cattle. Results are given at two cut-off values, *viz.* 14 PP and 18 PP. The application of both of these cut-off values in analysing the data leads to the incorporation of intermediate results between positive and negative threshold, for example <14 PP: negative; 14-18 PP: suspect; >18 PP: positive) (Greiner *et al.*, 1995). The interpretation of intermediate test results depends on the specific diagnostic purpose of the test. Re-testing individual animals, which may not be feasible in sero-epidemiological studies, was suggested for clarification of unexpected rather than intermediate test results (Worthington, 1993). For epidemiological studies, it is appropriate to consider only one cut-off value and indicate the test parameters for a given cut-off value selected because of the ambiguity of borderline results.

The overall specificity of the IgG Tamr-1 ELISA was high at both 14 PP (95.2%) and 18 PP (98.4%) cut-off values. The sensitivity of the test was low, i.e. 78.5% and 72.7% at 14 PP and 18 PP, respectively. However, if the cut-off value were chosen at 11 PP, rather than 14 PP, which corresponds to 90% specificity, the sensitivity of the test would be increased to 90.7%.

Both the sensitivity and specificity of serum samples used for IgG Tamr-1 ELISA were 100% in the piroplasm antigen IFAT. Thus, the specificity of the test was close to that of IFAT, whereas the sensitivity was substantially lower at both 14 and 18 PP cut-off values. Although the IFAT is more reliable then the Tamr-1 ELISA, Tamr-1 ELISA could be very useful in large scale sero-epidemiological studies as it is less laborious than IFAT.

In a preliminary study using a limited portfolio of control and test sera, the specificity and sensitivity of the Tamr-1 ELISA was previously reported to be 94% and 92%, respectively (Matita, 1994). This is in marked contrast to specificity and

sensitivity levels reported in the present study. Possible causes of differences between these two studies are i) number of samples tested, ii) the serum dilution used and iii) different cut-off values chosen.

In order to compensate for various factors that may influence the diagnostic sensitivity and specificity, the use of at least 300 known positive and 1000 known negative samples was suggested (Jacobson, 1996). However, it is very difficult to obtain such high numbers of animals under experimental conditions. In the study performed by Matita (1994), 33 known negative sera and 33 known positive sera from experimental calves infected on a single occasion with *T. annulata* sporozoites were used. The present study was conducted with significantly larger number of both positive and negative samples (188 negative, 209 positive).

Another reason for low sensitivity obtained in the current study with the Tamr-1 ELISA could be the optimisation of the test with a high affinity serum. Since high sensitivity is favoured by low serum dilutions (Venkatesan and Wakelin, 1993), this would result in choosing high serum dilutions and low antigen concentrations. The serum dilution used by Matita (1994) was 1:50 which is ten fold less than the one used in the present study. Thus, low affinity serum samples could not be detected.

The cut-off values chosen in the current study were in favour of specificity. When specificity of the test was 94% at the cut-off value of 13 PP, the sensitivity of the test was still lower than reported by Matita (1994). A simple reason for this could be that samples chosen to establish sensitivity and specificity of the test did not represent the field animal population in both experiments. The test sensitivity and specificity should not vary between studies if the animals chosen truly represent the population (Thrusfield, 1995).

The evaluation of the Tamr-1 antigen for cross-reactivity with other species of *Theileria* and other haemoprotozoan parasites indicated that cross-reactivity occurred with sera raised against *T. parva* as reported in other ELISAs performed with crude piroplasm antigens (Gray *et al.*, 1980; Kachani *et al.*, 1992). Cross reactivity between related species of *Theileria,* especially between *T. annulata* and *T. parva,* and other haemoparasites has been demonstrated in other serological tests (FAO, 1984) including IFAT (Burridge *et al.*, 1974). These cross-reactions are

important only where the distribution of the parasites may overlap as in the case of Southern Sudan where *T. annulata* and *T. parva* are both present (Lomuro, 1992).

A small proportion of serum samples obtained from animals infected with *T. buffeli*, *B. bovis* and *Tryp. evansi* also cross-reacted in the Tamr-1 ELISA. None of the serum samples from animals experimentally infected with *T. buffeli/T. sergenti* were positive. Only serum samples obtained from field infected animals from Sicily were found to be positive. It is evident from these observations that mixed infection with *T. annulata* and *T. buffeli* is probable. In fact, the presence of both *T. annulata* and *T. buffeli* in Sicily has been reported previously (Purnell, 1978). If the cross-reactivity between *T. annulata* and *T. buffeli* is due to shared epitopes, the use of the Tamr-1 ELISA in epidemiological studies is problematic, since the distribution of *T. buffeli* overlaps quite extensively with *T. annulata* (Mimioglu *et al.*, 1971; Fujisaki, 1992; Ceci *et al.*, 1997).

PP values of serum samples from animals infected with *B. bovis* and *Tryp. evansi* infected were inconclusive. More serum samples from *Trypanosoma* spp and *Babesia* spp infected animals should be tested to obtain unequivocal results since *T. annulata, Trypanosoma* spp and *Babesia* spp co-exist in many areas.

International standards for diagnostic tests for theileriosisis are lacking (Dolan, 1989). As a result, each laboratory validates diagnostic tests using "in house" materials. Since there is no "Gold Standard" test available for *T. annulata* infection, it is difficult to compare the results of different studies. To overcome this problem, a set of serum samples from experimentally infected animals were selected and re-coded. These serum samples were then made available to various laboratories to validate their ELISAs. The results of this blind test were analysed by a third person. At a cut-off value of 18 PP, the sensitivity of the blind test (75.0%) was very close to the estimated sensitivity for the standardisation of this test (72.7%) and there was no significant difference between these values. The blind test was carried out using Tams1-1 antigen by Gubbels *et al.*, (unpublished). The sensitivity and specificity of the Tams1-1 ELISA using these sera were 69.2% and 96.5% at a cut off value of 12.5 PP, respectively, indicating that the sensitivity obtained by the Tamr-1 ELISA appears to be better. However, the sensitivity and specificity of the Tamr-1 and Tams1-1 ELISAs were not significantly different ($p<0.05$).

In conclusion, the evidence provided in the current study indicates that Tamr-1 ELISA is not sensitive enough to replace the conventional IFAT, which is currently in use. However, the Tamr-1 ELISA could be a useful tool to study the large-scale sero-epidemiology of tropical theileriosis, though, it should be noted that the Tamr-1 ELISA is validated using serum samples from animals infected under controlled conditions that may not represent the actual field situation (Greiner *et al.*, 1997).

In the next chapter, results obtained with the recombinant antigens Tamr-1, NC10-Ssp13 and Tash-2 using serum samples from experimentally infected animals (Chapter 3) will be discussed. In these animal experiments, the field situation of both vaccination and field challenge were simulated to examine whether these stage–specific antigens could distinguish vaccinated animals from those that are naturally infected. Additionally, the duration of antibody responses to these antigens was examined.

CHAPTER SIX

VALIDATION OF ENZYME LINKED IMMUNO-SORBENT ASSAYS USING SERA FROM CALVES EXPERIMENTALLY INFECTED WITH *THEILERIA ANNULATA*

6.1. INTRODUCTION

The indirect fluorescent antibody test (IFAT) using both the macroschizont and piroplasm antigens is currently used as the standard serological assay to detect vaccinated and animals naturally infected with *T. annulata* (Pipano, 1974; Anon, 1997). The IFAT has contributed considerably to our understanding of the prevalence of theileriosis. It has also served as a valuable tool in monitoring experimental infections. However, the test has several drawbacks. For example, it is cumbersome to carry out and relies on subjective assessment of fluorescence. In addition, IFAT does not distinguish naturally infected animals from those that are vaccinated (Pipano *et al.*, 1969) because of the presence of common antigens in all life cycle stages of the parasite (Knight *et al.*, 1998). Detection of antibody responses before and after vaccination is required to determine if animals are exposed to the disease following vaccination. This is important to assess the efficacy of a vaccine. However, IFAT is not ideally suited to this purpose.

A natural infection with *T. annulata* sporozoites initiated by infected ticks results in the exposure of the host immune system to sporozoites, macroschizonts and merozoites/piroplasms. In contrast, animals immunised with an attenuated high passage schizont-infected cell line, the form of vaccine used widely in the control of theileriosis, which does not produce piroplasms, will be exposed only to the macroschizont stage of the parasite. Therefore, serological tests employing proteins specific to the sporozoite or piroplasm stages of the parasite in principle detect only animals infected with sporozoites, but not those vaccinated with macroschizonts. However, studies showed that animals infected with a single dose of sporozoites could not reliably be detected with a sporozoite surface antigen, SPAG-1, of *T. annulata* (Matita, 1994). This could be due to the fact that sporozoites are exposed to

host immune system for a short time period before they develop into macroschizonts (Jura *et al.*, 1983a).

Another approach to distinguish between vaccinated and naturally infected animals would be to use antigens specific to piroplasm stage of the parasite provided that the attenuated cell line vaccine does not produce piroplasms. To complement currently available piroplasm stage-specific antigens Tams1 (Shiels *et al.*, 1995) and Tamr-1 (Shiels *et al.*, 1994), a recombinant protein specific to the macroschizont stage of the parasite could be used to detect both animals infected with sporozoites and those that are vaccinated with an attenuated, high-passage cell line. The use of these two antigen-specific tests would provide the answers to the major questions concerning the epidemiology and control of tropical theileriosis viz. the prevalence of the disease and the effectiveness of attenuated cell line vaccines.

In this chapter, the stage-specific recombinant antigens, Tamr-1, NC10-Ssp13 and Tash-2, were screened against antisera produced as described in Chapter 3. The objectives of the current study were: i) to determine whether the Tamr-1 ELISA could detect animals infected with sporozoites but not those immunised with the high passage cell line vaccine; ii) to establish whether NC10-Ssp13 or Tash-2 antigens would detect both naturally infected and vaccinated animals; iii) to determine the period during which antibodies against Tamr-1, NC10-Ssp13 or Tash-2 antigens could be detected in the presence and absence of challenge; iv) to compare the results of ELISAs with the standard IFAT; v) to evaluate the Tamr-1 ELISA using serum samples obtained from Aydin province of Turkey where theileriosis is endemic. The last objective was also related to the carrier state of the animals as determined by piroplasm parasitaemia and PCR (Chapters 3 and 7).

6.2. MATERIAL AND METHODS

6.2.1. Serum Samples for ELISA and IFAT

6.2.1.1. Experimental infection of calves with T. annulata

Serum samples from animals that were experimentally infected either with sporozoites or with low and high passage cell lines were used. Experimental design, the clinical and haematological responses of animals were described in Chapter 3. Briefly, 21 calves were divided into three main groups referred to as Groups 1, 2 and 3 (Table 3.1). Calves in Group 1 (n=4) were inoculated with *T. annulata* Ankara sporozoites. Calves in Group 2 (Group 2A n=5; Group 2B n=4) were inoculated with *T. annulata* Ankara low-passage cell line. Calves in Group 3 (Group 3A n=4; Group 3B n=4) were inoculated with *T. annulata* Ankara/Pendik high-passage cell line in two consecutive experiments as described in Chapter 3. Animals in all groups were challenged with a heterologous *T. annulata* Gharb sporozoite stabilate either one or seven month(s) following the primary parasite inoculation.

6.2.1.2. Serum samples used for testing persistence of antibody responses against antigens representing specific stages of the parasite

Two groups of animals, Groups A and B, were tested for antibody responses against the Tamr-1, NC10-Ssp13 and Tash-2 recombinant antigens. Serum samples were tested only in IgG ELISAs.

Group A consisted of five calves; two Sahiwal heifers and three Friesian steers. These animals were infected with sporozoite stabilates and kept for 3 years without challenge. The three Friesian steers were then challenged with heterologous sporozoites. Table 6.1 shows the timing of infection and challenge. The serum samples that were tested are also given in Table 6.1.

Group B consisted of 11 calves that were infected with different parasite stocks at various doses of sporozoite stabilates. They were challenged on several occasions with a larger dose of sporozoites than that used for the initial infection Calves were monitored for 1 to 2.5 years (Table 6.2).

Table 6.1. Serum samples (Group A) used for testing the persistence of antibody responses against the recombinant antigens, Tamr-1, NC10-Ssp13 and Tash-2.

Calf No	Day post-infection	Parasite inoculation	Dose
488, 489	0	*Ta.* Hissar STAB 50, 52, 55, 62	0.1 t.e.
(Sahiwal)	28		
	57		
	84		
	365		
	3 years		
10A, 12A,	0	*Ta.* Hissar STAB 50, 52, 55, 62	0.1 t.e.
14A	28		
(Friesian)	3 years	*Ta.* Hissar STAB 50, 55, 62	4.0 t.e.
	Challenge 28		

Ta: T. annulata; t.e. tick equivalent; STAB: Stabilate; GUTS: ground up tick supernate

Table 6.2. Serum samples (Group B) used for testing the persistence of antibody responses against the recombinant antigens, Tamr-1 and Tash-2. Days in brackets indicate the day after challenge(s).

Calf No	Day post-infection (challenge)	Parasite inoculation	Dose/t.e.
891,900, 892, 899	0 (pre-infection)	*Ta* Hissar STAB 26,37,43,46,49	0.1
	34	*Ta* Gharb STAB 44,45,47	4.0
	63(30)		
	150(117)		
	216(183)		
	245(212,28)	*Ta* Ankara STAB 13,22B,23,48	1.6
	354(321,137)		
	413(380,196)		
11434	0 (pre-infection)	*Ta* Hissar STAB 52	2.0
	28		
	57		
	108	*Ta* Hissar GUTS	20.2
	119		
	122		
	140		
	588		
1052,1055	0 (pre-infection)	*Ta* Ankara STAB 48	2.0
	28		
	88		
	133 (25)	*Ta* Hissar GUTS	20.0
	156 (48)		
	218(62)		
	525(416)		
	532(423)	*Ta* Hissar GUTS	20.0
	553(444,17)		
	564(455,28)		
	791(682,255)		
	884(775,348)	*Ta* Hissar GUTS	20.0
	903(794,367,10)		
	906(797,370,13)		
1219, 902, 903, 120A	0 (pre-infection)	*Ta* Hissar STAB 26,37,43,46,49	0.001
	28		
	58	*Ta* Gharb STAB 44,45,47	4.0
	86(28)		
	150(120,92)		
	268(238,210)		
	428(398,270)		
	459(429,301)	*Ta* Ankara STAB 13,22B,23,48	1.6
	487(457,329,28)		

Ta: T. annulata; t.e. tick equivalent; STAB: Stabilate; GUTS: ground up tick supernate

6.2.1.3. Field serum samples

A total of 79 serum samples collected from cattle with a history of theileriosis around the Aydin province of Turkey, where tropical theileriosis is endemic (see section 7.2.1.5), were tested by IFAT using both piroplasm and macroschizont antigens and by the IgG Tamr-1 ELISA. These cattle were also examined for the presence of piroplasms by Giemsa's stained blood smears and PCR as described in Chapter 7. The results obtained from ELISA, IFAT, blood smear and PCR were analysed using the Chi-square distribution (Minitab, version 10.2).

6.2.2. ELISA

6.2.2.1. Recombinant antigens

To investigate the diagnostic significance of isotype marker in theileriosis, levels of specific IgG and IgM were analysed using Tamr-1, NC10-Ssp13 and Tash-2 recombinant antigens.

6.2.2.2. ELISA protocol

The ELISA protocols for both IgG and IgM ELISAs and their standardisation were described in Chapter 5.

6.2.2.3. Data expression and cut-off values

The data obtained from the IgG Tamr-1 ELISA were expressed as percentage positivity (PP) as described in Chapter 5. The data obtained from the IgM Tamr-1, IgG and IgM Tash-2 and NC10-Ssp13 ELISAs were expressed as absorbance value.

Cut off values of 14 PP and 18 PP were chosen for the IgG Tamr-1 ELISA based on the standardisation of this ELISA as described in Chapter 5. For the IgM Tamr-1 ELISA and the IgG and IgM ELISAs of NC10-Ssp13 and Tash-2, pre-infection serum samples (day 0) were accepted as reference. The positive/negative cut-off lines for these ELISAs were set as two times the absorbance value of pre-infection serum for each calf (de Savigny and Voller, 1980). In order to avoid plate to plate variations, sequential serum samples obtained from each animal were tested on the same ELISA plate and antibody responses were correlated with the corresponding pre-infection values.

6.2.3. IFAT

6.2.3.1. Antigen preparation

i) Macroschizont antigen

Antigen slides were prepared from cultures as described previously (Goddeeris *et al.*, 1982; Minami *et al.*, 1983). Briefly, cells obtained from a 2 day-old *T. annulata* culture were centrifuged at 300 x g for 10 minutes at 4°C, the supernate discarded and cells were washed in cold PBS three times. Before the last wash, the cell viability and infection rates were assessed. Viability and infection rates both of 95 % were considered optimal. Cells were resuspended to approximately 5 x 10^7 cells/ml in cold PBS and an equal volume of cold 1:10 formalin (3.7 % formaldehyde, Analar, BDH) was added to the cell suspension dropwise while gently stirring the tube. Cells were fixed on ice for 10 minutes, washed in PBS three times, and resuspended to 2 x 10^7 cells/ml in PBS. Antigen slides were prepared by placing a 20 µl volume of cell suspension onto each well of multiwell slides (ICN Biomedicals Ltd). Cells were removed immediately using an automatic pipettor to leave a thin layer of cells on each well. Slides were air-dried at room temperature and wrapped in tissue paper and stored in self-sealing polythene bags containing silica gel (BDH) at –20°C.

ii) Piroplasm antigen

Piroplasm antigen slides were prepared as described previously (Burridge, 1971). Approximately 5 ml of blood was collected directly into 150 ml of cold PBS from the jugular vein of a calf, experimentally infected with *T. annulata* Hissar, when the parasitaemia was 82%. The blood sample was centrifuged at 300 x g for 10 minutes at 4°C. The supernatant was discarded along with the buffy coat. Erythrocytes were washed three times in PBS and centrifuged at 300 x g for 10 minutes at 4°C. The supernatant was discarded along with the buffy coat. Following the last wash, erythrocytes were resuspended in 5 ml PBS supplemented with 1% bovine plasma albumin (Sigma) at 1:20 (v/v). Multiwell antigens were prepared as described above for the macroschizont antigens.

6.2.3.2. IFAT method

Serum samples from animals infected experimentally (section 6.2.1.1) and from animals in the field (see section 6.2.1.3) were tested using both macroschizont and piroplasm antigens. Antigen slides were thawed at 4°C for 30 minutes in a sealed bag with silica gel and kept at room temperature for another 30 minutes. Acetone fixed antigen slides were rinsed in PBS four times (10 minutes each), then air-dried at room temperature. Formalin fixed slides were ready to use after thawing. Meanwhile four-fold dilutions of sera from 1:40 to 1:2560 were prepared in PBS.

Ten microlitres of the serum dilutions were placed into wells of antigen slides. Each antigen slide included the following controls: *T. annulata* positive and negative sera diluted at 1:160 in PBS and PBS alone as a conjugate control. Slides were incubated in a moist chamber for 30 minutes at room temperature, washed in PBS three times (10 minutes each) and air-dried at room temperature. Rabbit anti-bovine IgG conjugated to fluorescein isothiocyanate (FITC) (Sigma) at a dilution of 1:80 in PBS was placed onto each well. The PBS contained 0.01% of Evans Blue (Sigma) which served as counterstain. Slides were incubated in the moist chamber in the dark for 30 minutes, then washed in PBS three times (10 minutes each). The slides were air-dried, mounted in 66% (v/v) glycerol in 50 mM Tris-HCl, pH 9.2, using 22 x 40 mm coverslips. Slides were examined for fluorescence under x 20 or x 40 oil immersion objective using an blue violet incident light fluorescence microscope (Leitz Orthoplan, Wetzlar). The degree of fluorescence was recorded as strong fluorescence (+) (positive reaction), weak positive (+/-) or no fluorescence (-) (negative reaction). Only strong fluorescence (+) was accepted as positive. The weak positive fluorescence (+/-) and no fluorescence (-) were considered to be negative. The IFAT titre of 1:160 was established as cut-off value for the positivity of serum samples.

6.3. RESULTS

6.3.1. ELISA Results of Calves Immunised against *T. annulata* by Infection with Different Parasite Stages

Serum samples from animals that were experimentally infected either with sporozoites or with low and high passage cell lines (see section 6.2.1.1) were tested by both the IgG and IgM ELISAs using Tamr-1, NC10-Ssp13 and Tash-2.

6.3.1.1. Immunoglobulin G response of experimentally infected animals

Results of IgG ELISAs using Tamr-1, NC10-Ssp13 and Tash-2 are given in Figures 6.1-6.3 respectively.

i) IgG Tamr-1 ELISA

All the calves had PP values below 14 PP prior to infection with sporozoites or macroschizont infected cell lines. The profiles of the IgG response of calves in Group 1 using the Tamr-1 ELISA was not reproducible as two calves had low and the other two had high antibody titres. The response of calf 21A increased to above the cut-off points earlier than other calves in this group (Figure 6.1A). The PP value of calf 21A was above the cut-off points by day 35. It remained as such until challenge with *T. annulata* Gharb sporozoites 7 month after primary infection. Calf 23B had the lowest antibody response in Group 1. PP values for this animal were above the cut-off values by day 85 and fluctuated thereafter around the cut-off values until challenge (Figure 6.1A). The response of the other two calves in this group was similar to each other. PP values were detectable between days 42 and 56 and remained above or very close to cut off values until challenge.

PP values of calves in Group 2A immunised with *T. annulata* Ankara low passage cell line were high in the Tamr-1 ELISA (Figure 6.1B). All animals in this group responded to infection relatively early. IgG antibodies could be detected at 14 or 21 days post-immunisation. Antibody response remained above cut-off values throughout the monitoring period, with the exception of calf 24B. The IgG response of calf 24B was detected only on day 21, and its PP values remained below cut-off values after challenge (Figure 6.1B).

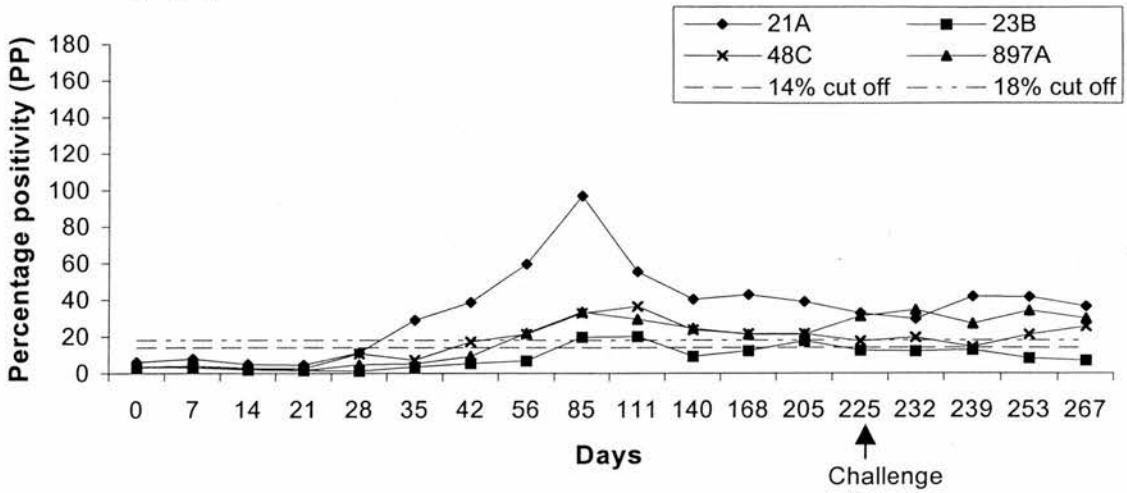
Figure 6.1. IgG responses of calves infected with *T. annulata* Ankara sporozoites or immunised with the high or low passage cell lines detected by Tamr-1 ELISA. Calves were challenged with a heterologous stock of *T. annulata* Gharb sporozoite stabilate either 35 days or 7 months following the primary parasite inoculation.

A. Group 1: Calves infected with *T. annulata* Ankara sporozoites and challenged 7 months after primary infection.

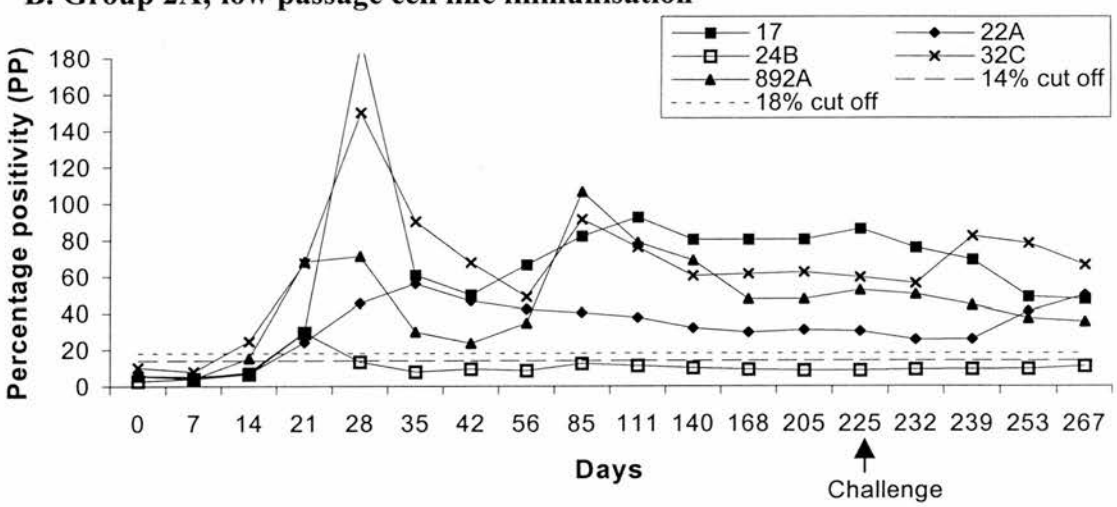
B. Group 2A: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 7 months after primary infection.

C. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 7 months after primary infection

A. Group 1; sporozoite infection



B. Group 2A; low passage cell line immunisation



C. Group 3A; high passage cell line immunisation

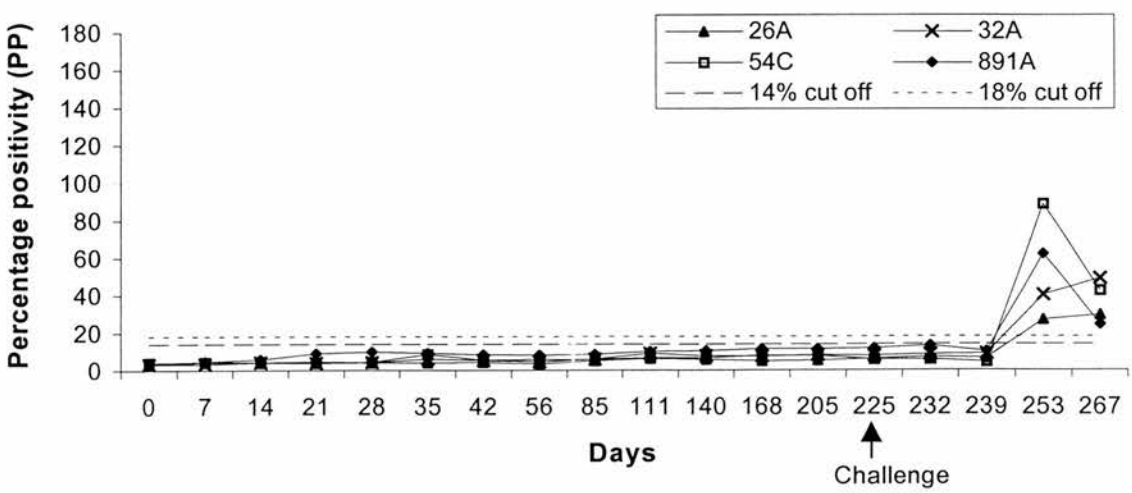
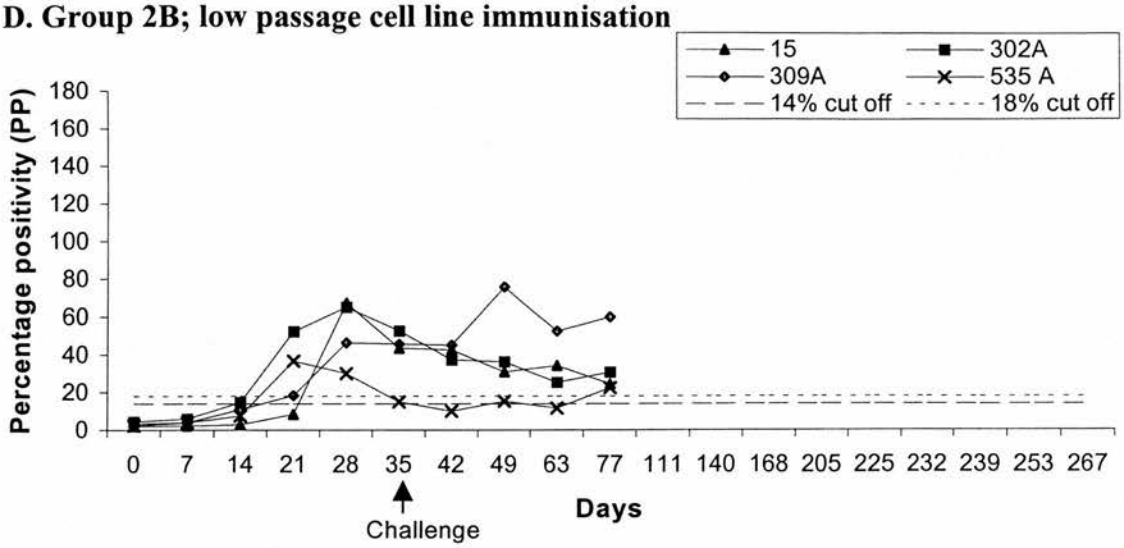


Figure 6.1. Continued

D. Group 2B: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 35 days after primary infection.

E. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 35 days after primary infection.

D. Group 2B; low passage cell line immunisation



E. Group 3B; high passage cell line immunisation

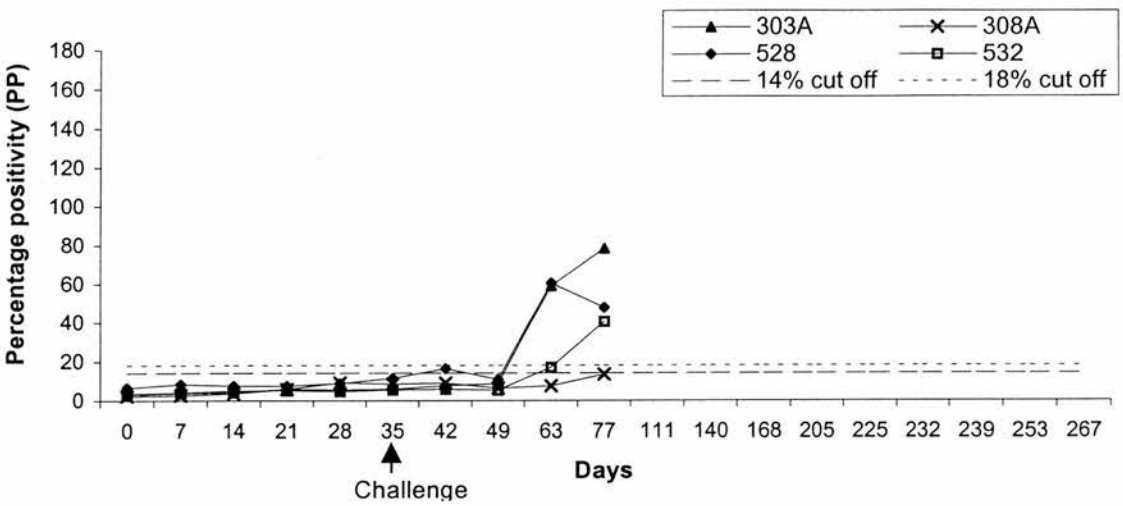


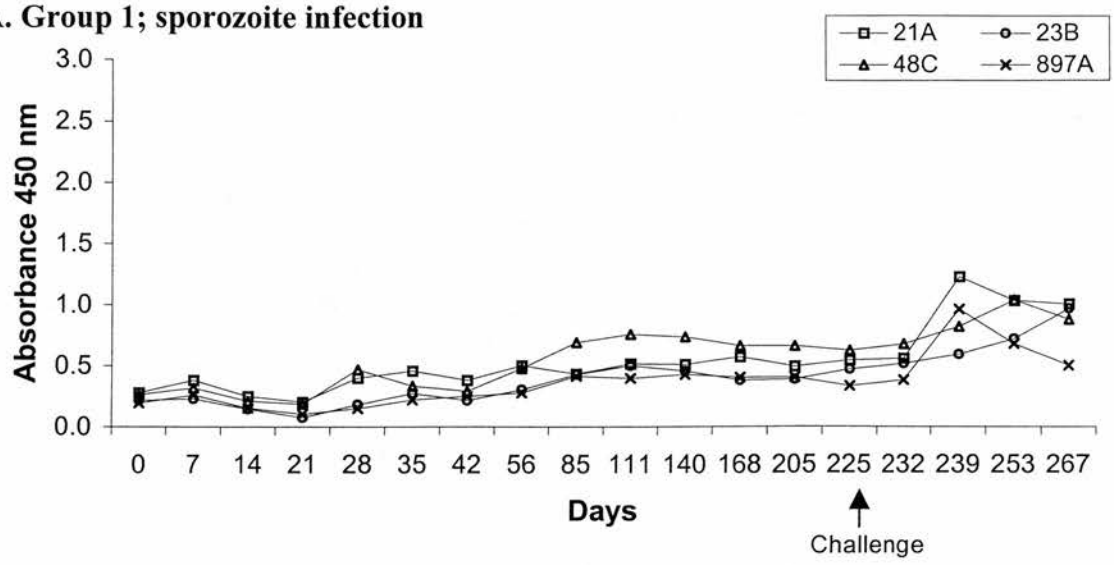
Figure 6.2. IgG responses of calves infected with *T. annulata* Ankara sporozoites or immunised with the high or low passage cell lines detected by NC10-Ssp13 ELISA. Calves were challenged with a heterologous stock of *T. annulata* Gharb sporozoite stabilate either 35 days or 7 months following the primary parasite inoculation.

A. Group 1: Calves infected with *T. annulata* Ankara sporozoites and challenged 7 months after primary infection.

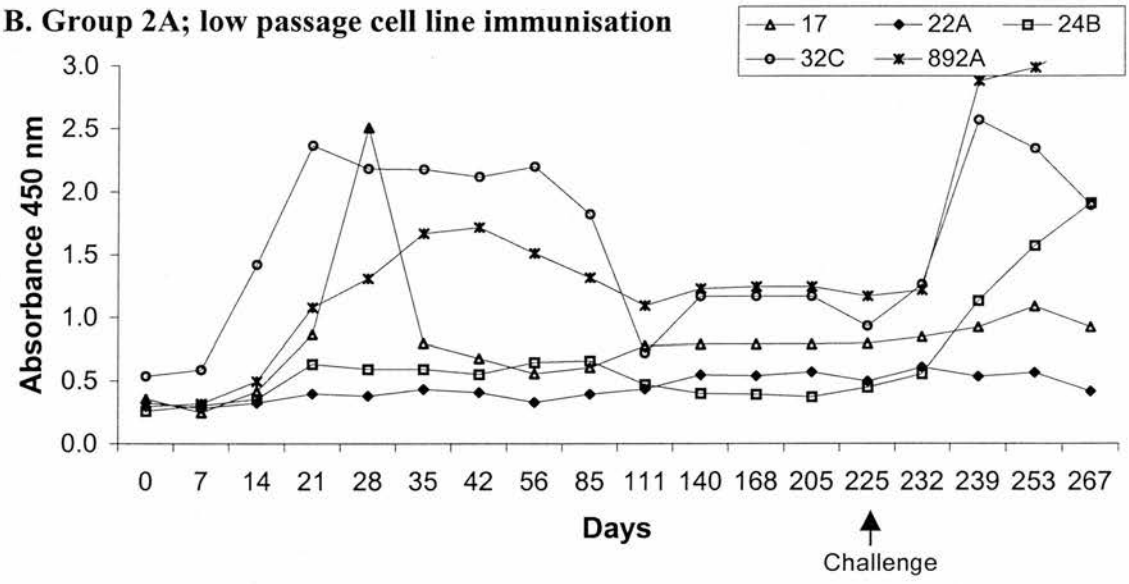
B. Group 2A: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 7 months after primary infection.

C. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 7 months after primary infection

A. Group 1; sporozoite infection



B. Group 2A; low passage cell line immunisation



C. Group 3A; high passage cell line immunisation

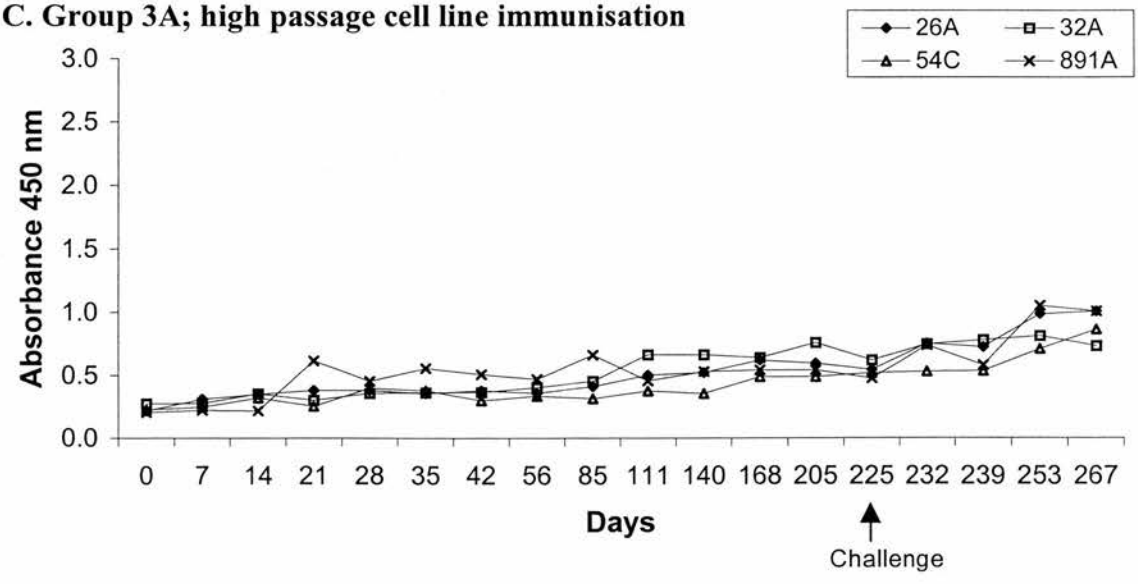
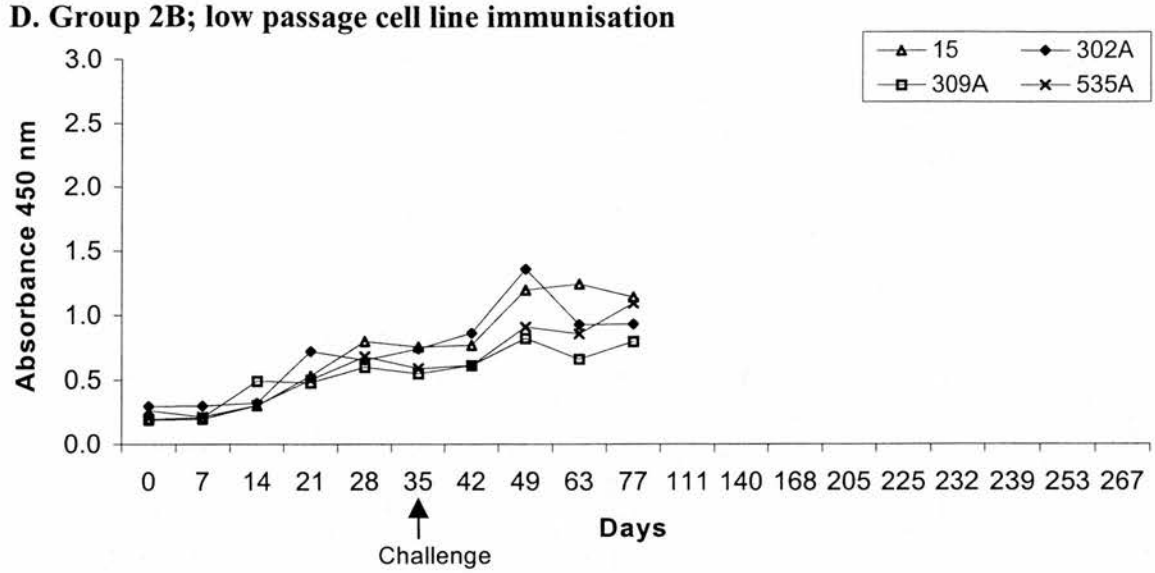


Figure 6.2. Continued

D. Group 2B: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 35 days after primary infection.

E. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 35 days after primary infection.

D. Group 2B; low passage cell line immunisation



E. Group 3B; high passage cell line immunisation

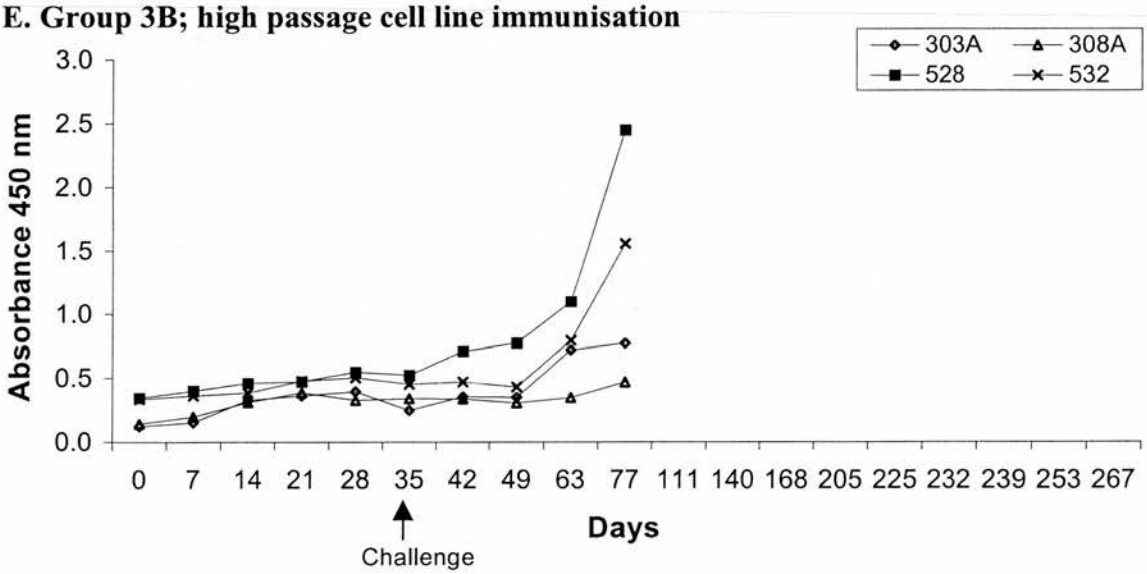


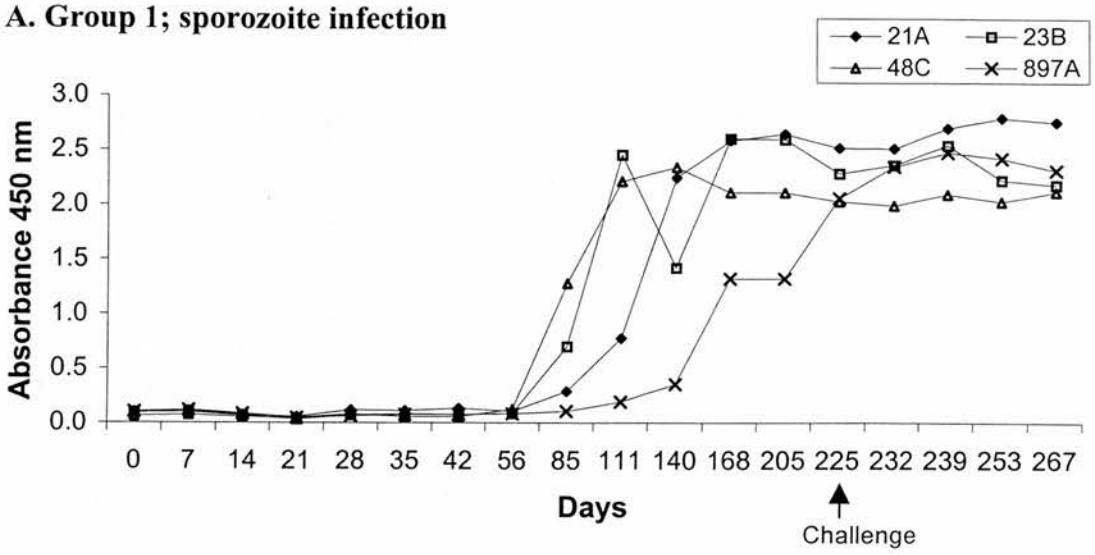
Figure 6.3. IgG responses of calves infected with *T. annulata* Ankara sporozoites or immunised with the high or low passage cell lines detected by Tash-2 ELISA. Calves were challenged with a heterologous stock of *T. annulata* Gharb sporozoite stabilate either 35 days or 7 months following the primary parasite inoculation.

A. Group 1: Calves infected with *T. annulata* Ankara sporozoites and challenged 7 months after primary infection.

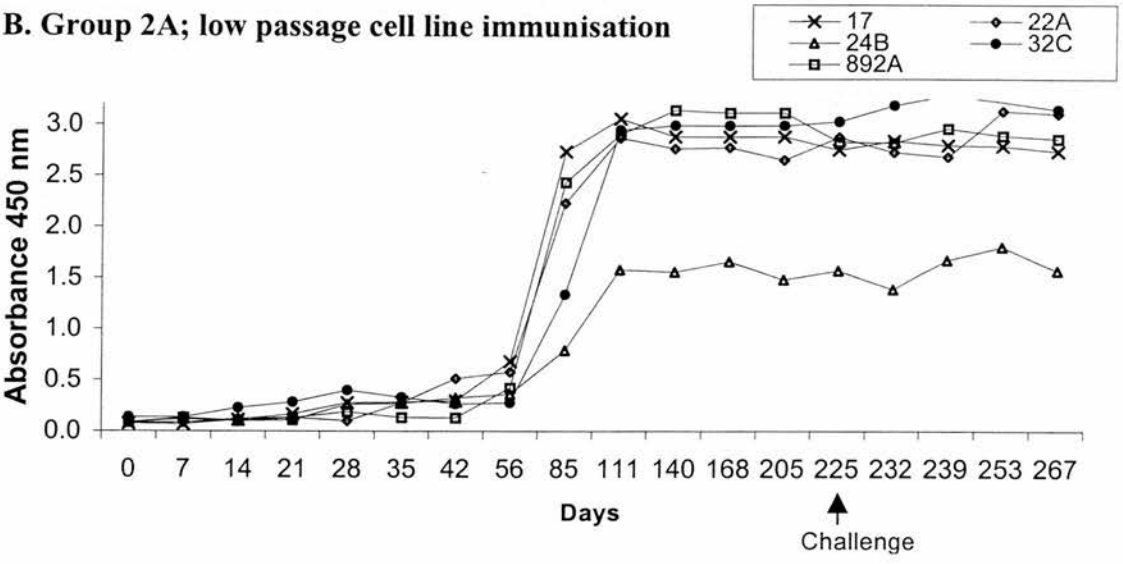
B. Group 2A: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 7 months after primary infection.

C. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 7 months after primary infection

A. Group 1; sporozoite infection



B. Group 2A; low passage cell line immunisation



C. Group 3A; high passage cell line immunisation

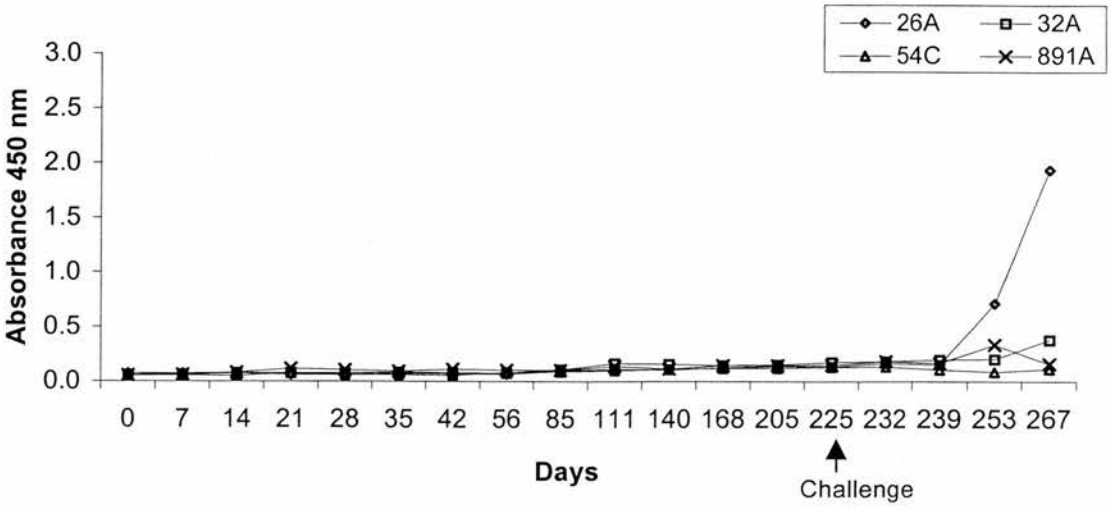
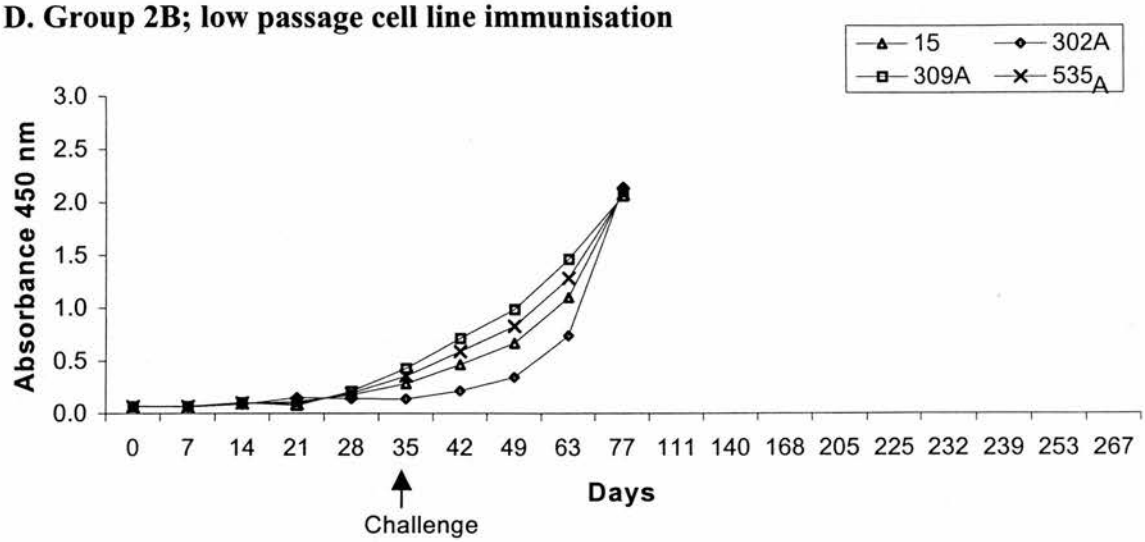


Figure 6.3. Continued

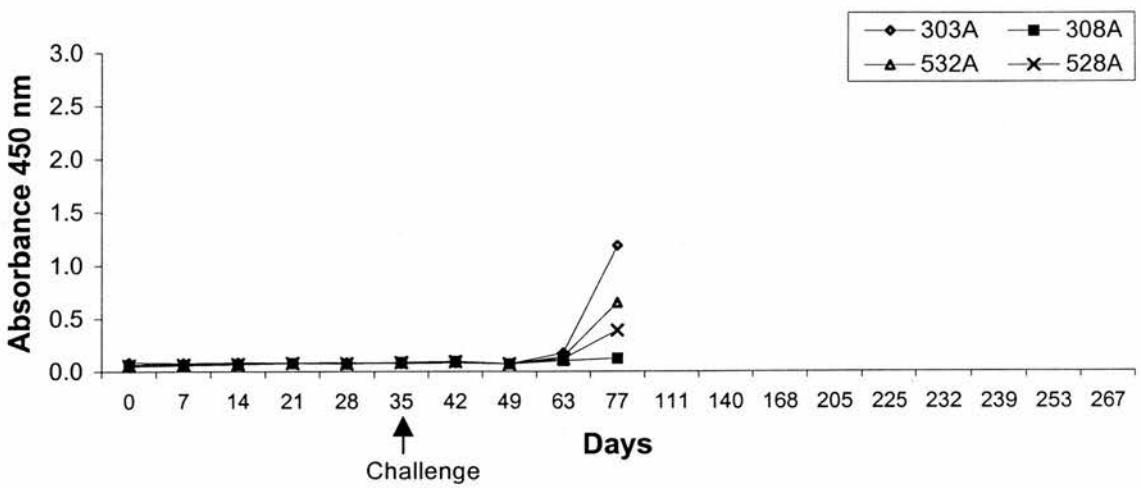
D. Group 2B: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 35 days after primary infection.

E. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 35 days after primary infection.

D. Group 2B; low passage cell line immunisation



E. Group 3B; high passage cell line immunisation



The early antibody response detected in this group was coincident with the early appearance of piroplasms on day 7 (see Chapter 3). Calves in Group 2A showed two antibody peaks. The first peak was between days 21 and 35 and the second peak between days 84 and 112. The second peak coincided with the antibody peak observed in Group 1. PP values of three out of five animals in Group 2A did not increase following challenge 7 months after the primary immunisation.

Calves in Group 2B which were immunised with the low passage cell line and challenged 35 days later had antibody responses similar to that described for Group 2A. All calves had detectable and peak IgG responses between days 21 and 28 (Figure 6.1D). Following challenge, the antibody level of calf 309 continued to increase while those of other calves remained relatively similar to pre-challenge levels.

All calves in Groups 3A and 3B had low antibody responses both before and after primary immunisation (Fig 6.1C and E respectively). PP values of animals within these groups were below the cut-off values throughout the infection. All calves, except calf 308, had detectable levels of antibody by day 42 following challenge.

ii) IgG NC10-Ssp13 ELISA

The pattern of antibody profiles was very similar in all calves in Group 1. All calves showed slight, yet continuous, increase in IgG antibody responses during the course of infection. Antibody levels reached above twice the pre-infection OD levels between days 85 and 168 following primary infection in all calves (Figure 6.2A).

There were considerable differences in the antibody responses to primary infection and challenge among calves in Group 2A. In four out of five calves, antibody levels reached to above twice the pre-infection OD values after immunisation (Figure 6.2B). The antibody response of these animals fluctuated before challenge. Following challenge, antibody response in these four calves increased above twice the pre-infection OD level (Figure 6.2B). One calf (calf 22A) did not show any antibody response to infection and challenge.

Antibody response of calves in Group 2B, increased to twice the pre-infection OD levels within 14 days following the immunisation and steadily increased until and after challenge (Figure 6.2D).

Calves in Group 3A had similar antibody responses to those in Group 1. IgG antibody levels were low but steadily increased from 7 or 21 days post-immunisation until and after challenge. The antibody response in all calves reached twice the pre-infection OD levels by days 21 or 168 (Figure 6.2C).

Antibody response of two out of four calves in Group 3B increased to twice the pre-infection OD levels by day 14. Antibody level of the remaining two calves reached twice the pre-infection OD levels only following challenge (Figure 6.2E).

iii) IgG Tash-2 ELISA

Results obtained from the IgG Tash-2 ELISA are demonstrated in Figure 6.3A-E. Calves in Group 1 showed less variation in their IgG response to Tash-2 than the IgG response to Tamr-1. All calves in Group 1 showed a greater antibody response to primary infection than that obtained using the Tamr-1 and NC10-Ssp13 antigens. Following infection, the antibody response remained around the base line until day 56 (calves 21A, 23B and 48C) or day 85 (897A) and then quickly increased to a very high level (Figure 6.3A). The antibody level remained high throughout the secondary infection *i.e.* after challenge.

Groups 2A and 2B had antibody profiles similar to that described for Group 1 except that the antibody response was earlier and at a higher level (Figure 6.3B and D). Antibody levels increased to a high level by days 21-42 after immunisation and remained high throughout the primary immunisation and after challenge.

There was only a slight increase in IgG levels to Tash-2 antigen in Groups 3A and 3B after immunisation (Figure 6.3C and E). The antibody levels of three calves in group 3A reached twice the pre-infection OD levels by days 111 or 140 (Figure 6.3C). Following challenge, all calves, except 54C and 308A, showed an antibody response at different levels.

6.3.1.2. Immunoglobulin M response of experimentally infected animals

Results of IgM ELISAs using Tamr-1, NC10-Ssp13 and Tash-2 are given in Figures 6.4-6.6 respectively.

i) IgM Tamr-1 ELISA

A classic IgM response was obtained following infection with sporozoites in Group 1 (Figure 6.4A). The IgM response occurred before the IgG response. IgM levels showed an increase in two calves by day 21 and remained high until day 85. Antibody levels declined before challenge in all animals except calf 48C. There was a slight increase in antibody levels in some animals following challenge (Figure 6.4A).

The IgM responses of Group 2A were greater and more rapid than those of Group 1 (Figure 6.4B). Antibody titres in all calves reached twice the pre-infection OD values by days 7 and 21 following immunisation. Antibody levels declined almost to pre-infection levels between days 46 and 168 in three out of five calves (Figure 6.4B).

Antibody responses of calves in Group 2B were similar to those of Group 2A. Antibody levels peaked on day 21 or 28. A rapid decline was observed until and following challenge with the exception of calf 15, which had a peak antibody response 15 days following challenge (Figure 6.4D).

Calves in Group 3A had only a slight increase in IgM levels after the immunisation (Figure 6.4C). Following challenge, antibody titres increased rapidly, reaching a peak on day 28 after challenge and declined afterwards.

The IgM titre increased to twice the pre-infection levels in two calves (303A, 308A) after immunisation in Group 3B. In the remaining calves, the IgM responses were detected only following challenge (Figure 6.4E).

ii) IgM NC10-Ssp13 ELISA

Results obtained from the IgM NC10 Ssp-13 ELISA are illustrated in Figure 6.5 A-E. IgM antibody profiles of individual calves in Group 1 were similar to those observed with the IgG antibody for the same group. Antibody titres reached twice the

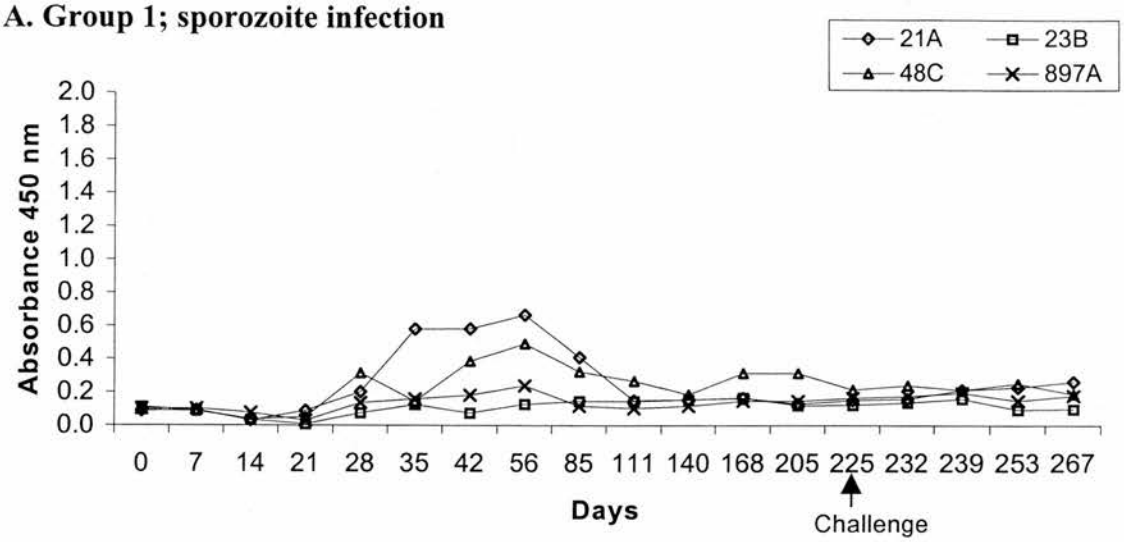
Figure 6.4. IgM responses of calves infected with *T. annulata* Ankara sporozoites or immunised with the high or low passage cell lines detected by Tamr-1 ELISA. Calves were challenged with a heterologous stock of *T. annulata* Gharb sporozoite stabilate either 35 days or 7 months following the primary parasite inoculation.

A. Group 1: Calves infected with *T. annulata* Ankara sporozoites and challenged 7 months after primary infection.

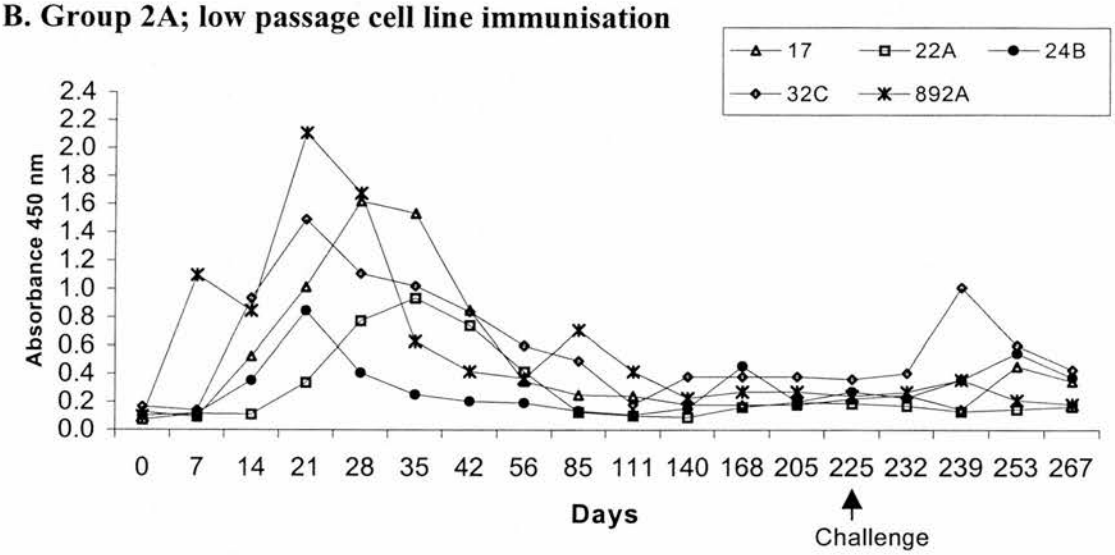
B. Group 2A: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 7 months after primary infection.

C. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 7 months after primary infection

A. Group 1; sporozoite infection



B. Group 2A; low passage cell line immunisation



C. Group 3A; high passage cell line immunisation

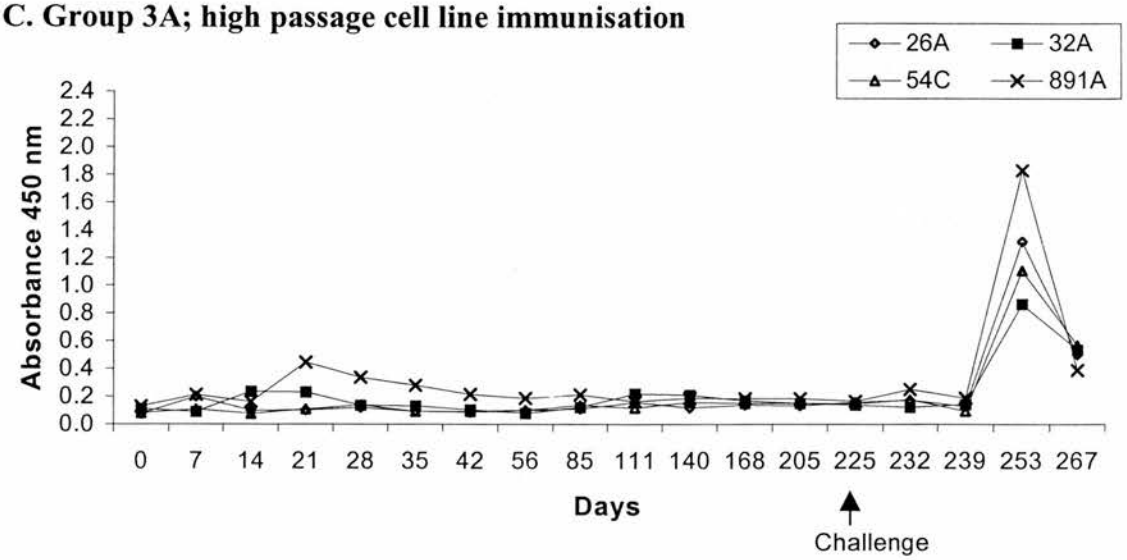
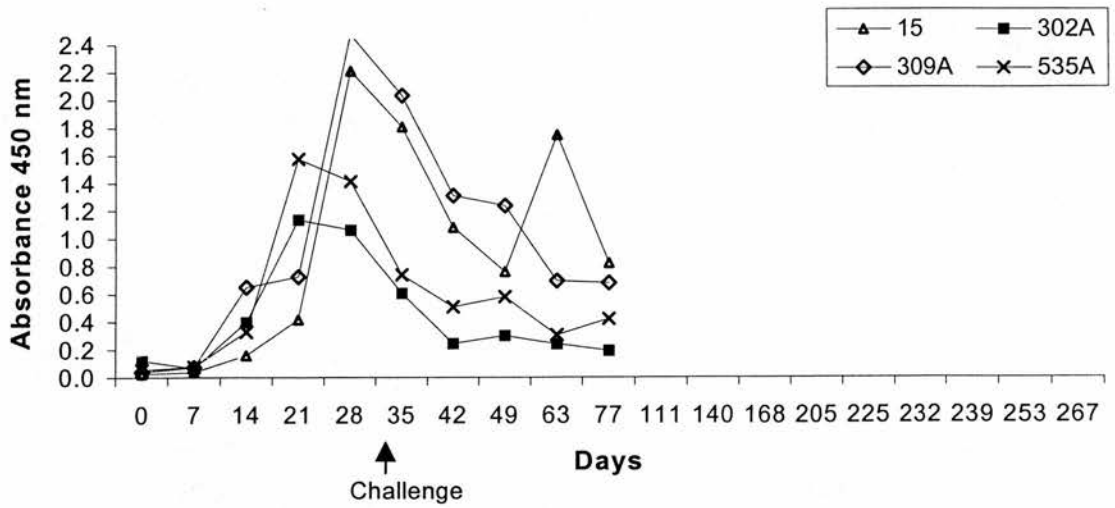


Figure 6.4. Continued

D. Group 2B: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 35 days after primary infection.

E. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 35 days after primary infection.

D. Group 2B; low passage cell line immunisation



E. Group 3B; high passage cell line immunisation

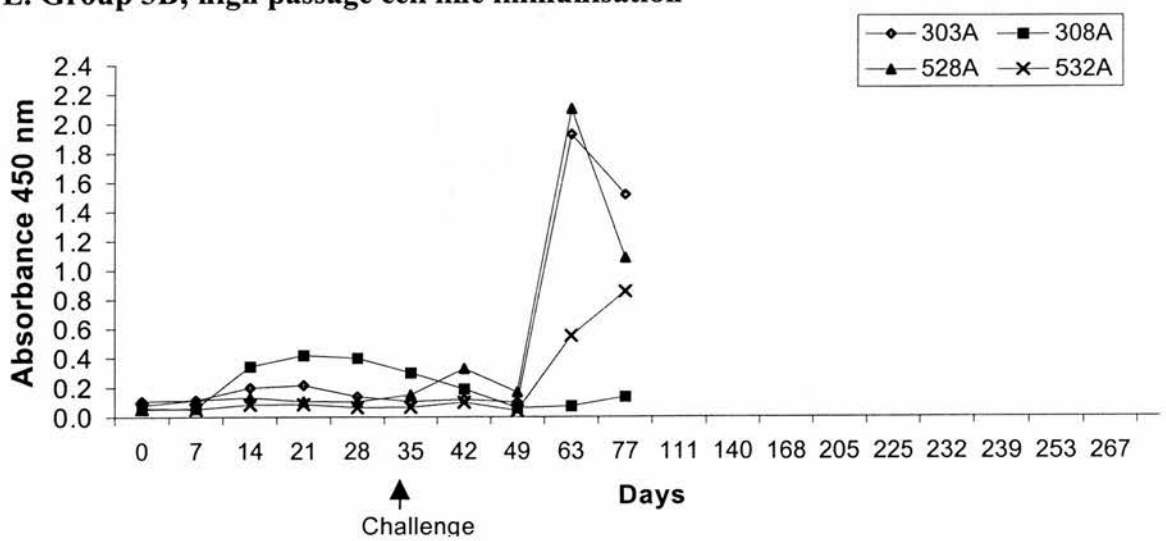


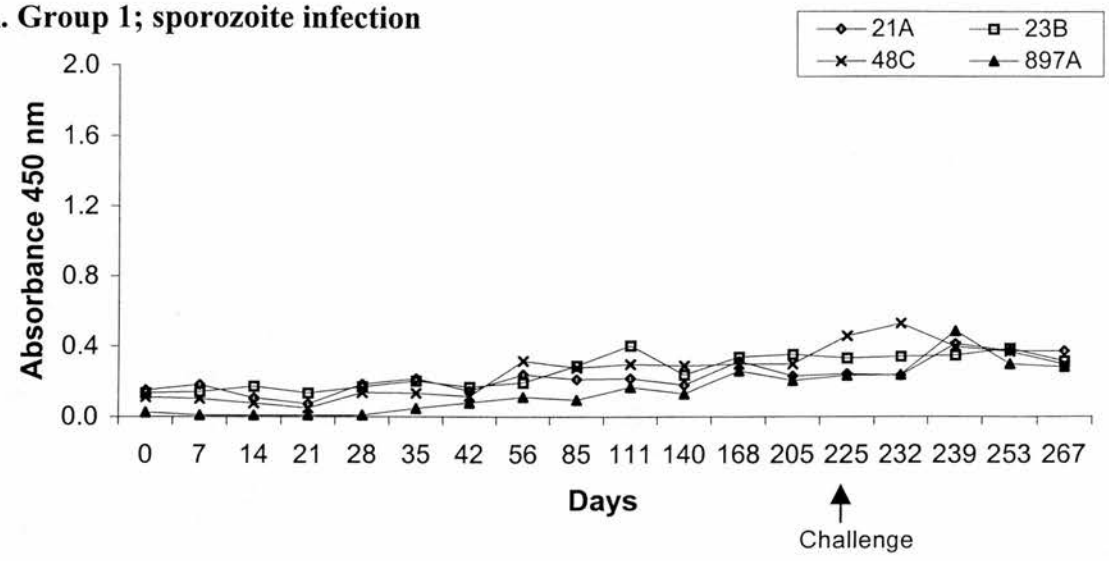
Figure 6.5. IgM responses of calves infected with *T. annulata* Ankara sporozoites or immunised with the high or low passage cell lines detected by NC10-Ssp13 ELISA. Calves were challenged with a heterologous stock of *T. annulata* Gharb sporozoite stabilate either 35 days or 7 months following the primary parasite inoculation.

A. Group 1: Calves infected with *T. annulata* Ankara sporozoites and challenged 7 months after primary infection.

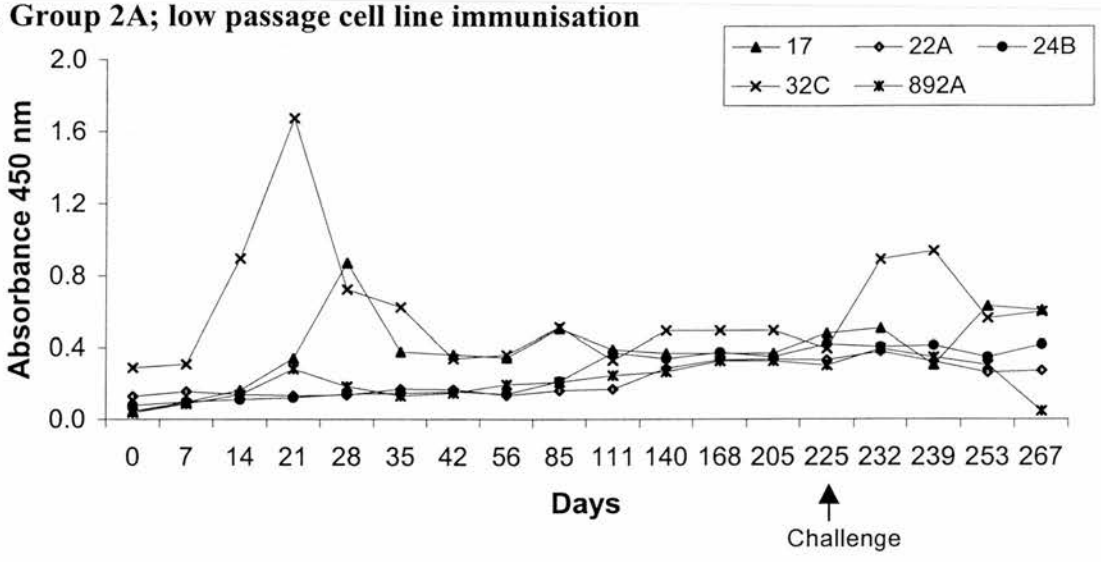
B. Group 2A: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 7 months after primary infection.

C. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 7 months after primary infection

A. Group 1; sporozoite infection



B. Group 2A; low passage cell line immunisation



C. Group 3A; high passage cell line immunisation

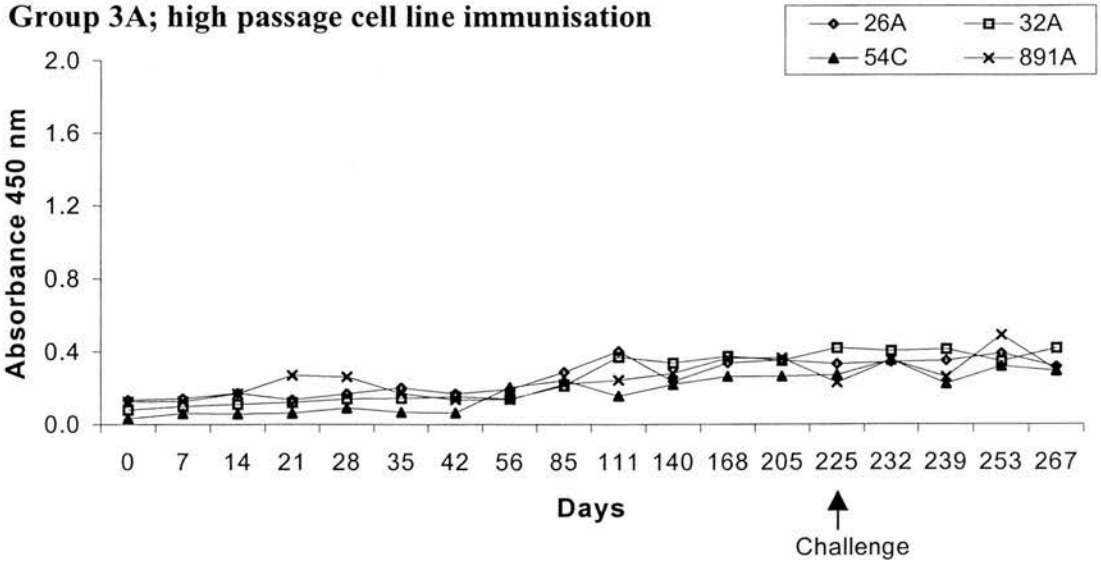
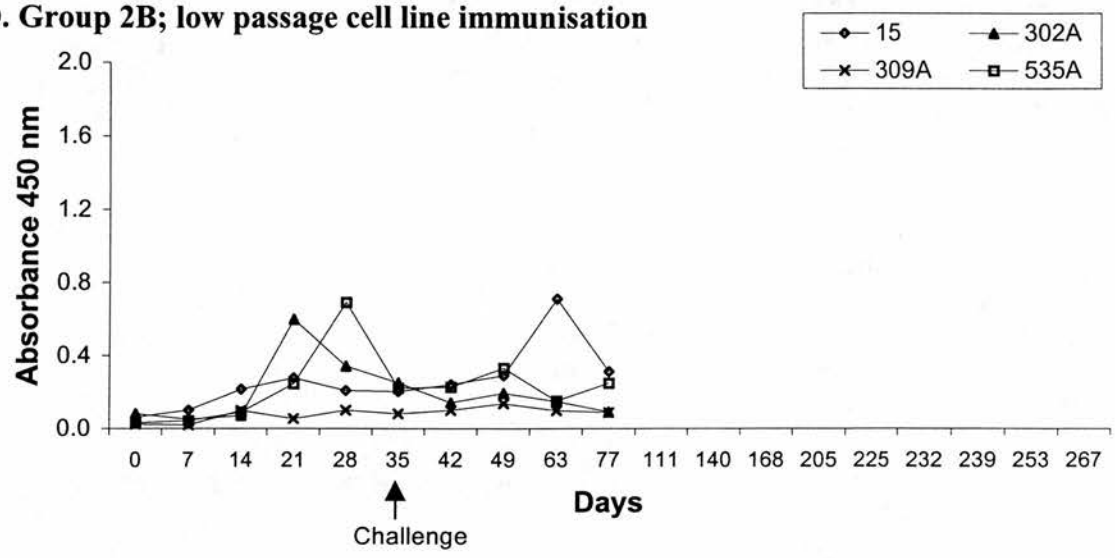


Figure 6.5. Continued

D. Group 2B: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 35 days after primary infection.

E. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 35 days after primary infection.

D. Group 2B; low passage cell line immunisation



E. Group 3B; high passage cell line immunisation

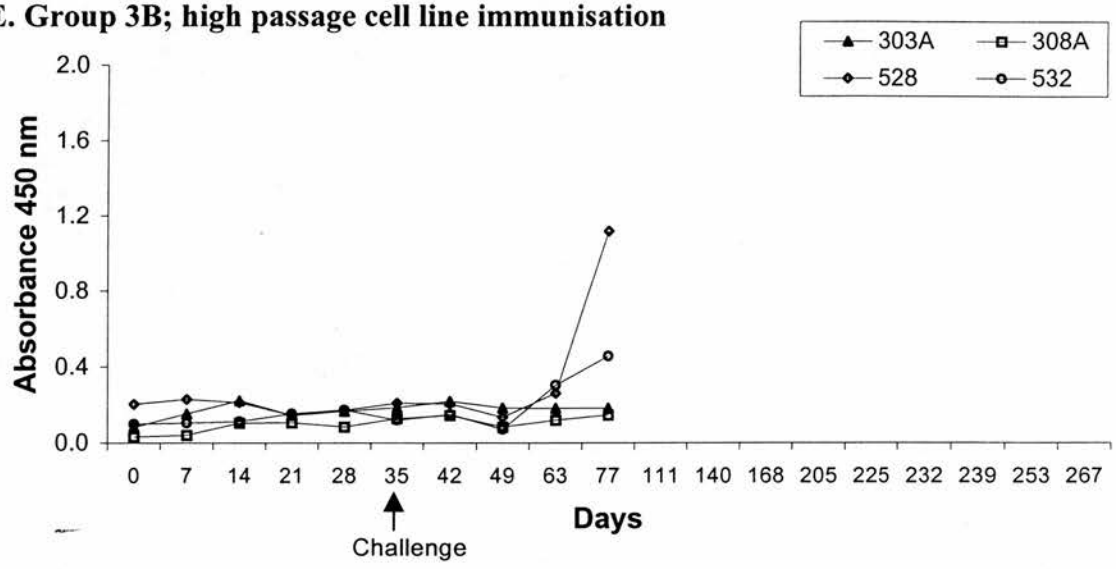


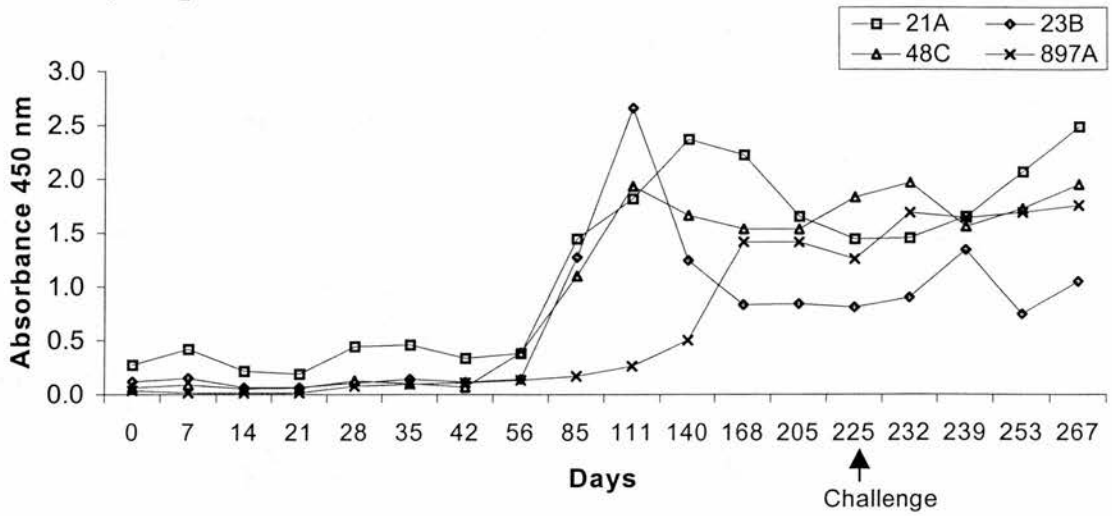
Figure 6.6. IgM responses of calves infected with *T. annulata* Ankara sporozoites or immunised with the high or low passage cell lines detected by Tash-2 ELISA. Calves were challenged with a heterologous stock of *T. annulata* Gharb sporozoite stabilate either 35 days or 7 months following the primary parasite inoculation.

A. Group 1: Calves infected with *T. annulata* Ankara sporozoites and challenged 7 months after primary infection.

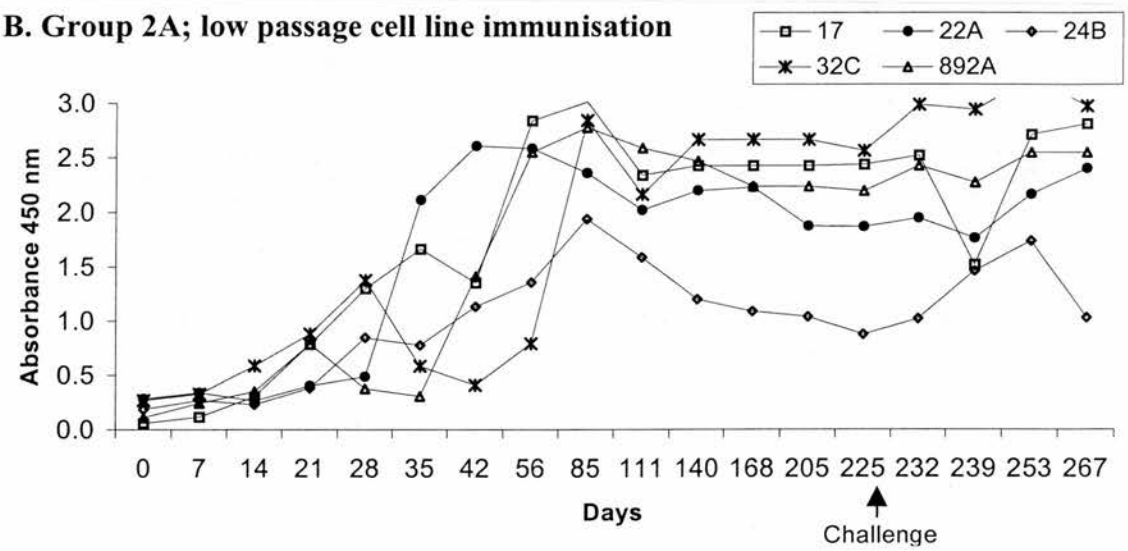
B. Group 2A: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 7 months after primary infection.

C. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 7 months after primary infection

A. Group 1; sporozoite infection



B. Group 2A; low passage cell line immunisation



C. Group 3A; high passage cell line immunisation

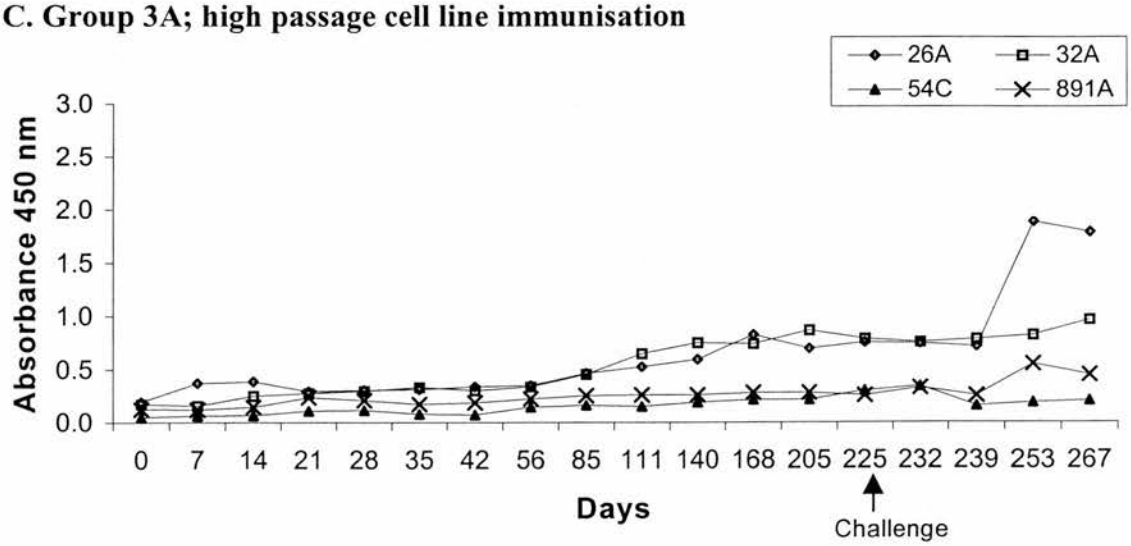
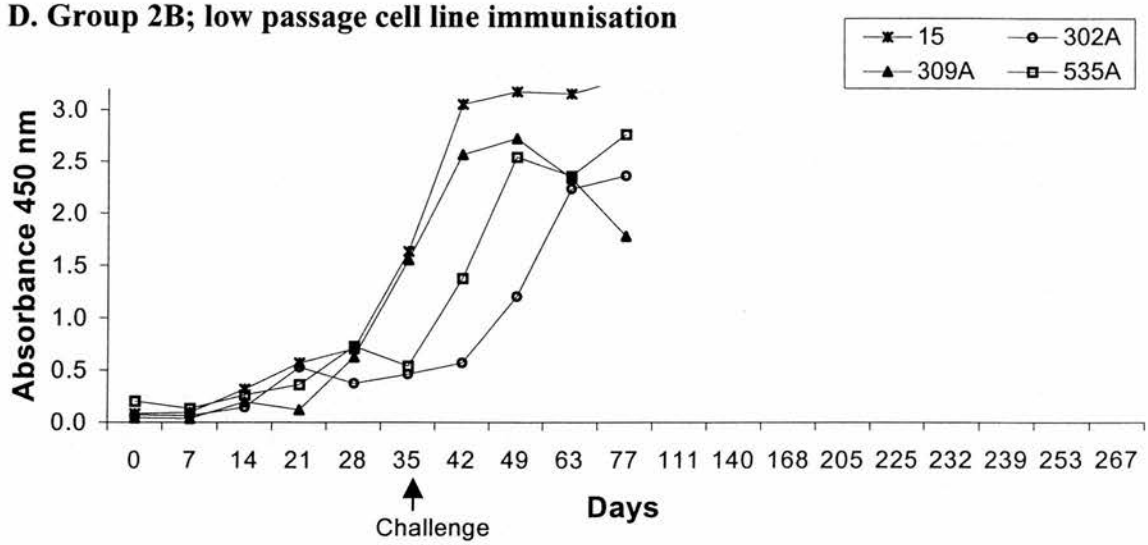


Figure 6.6. Continued

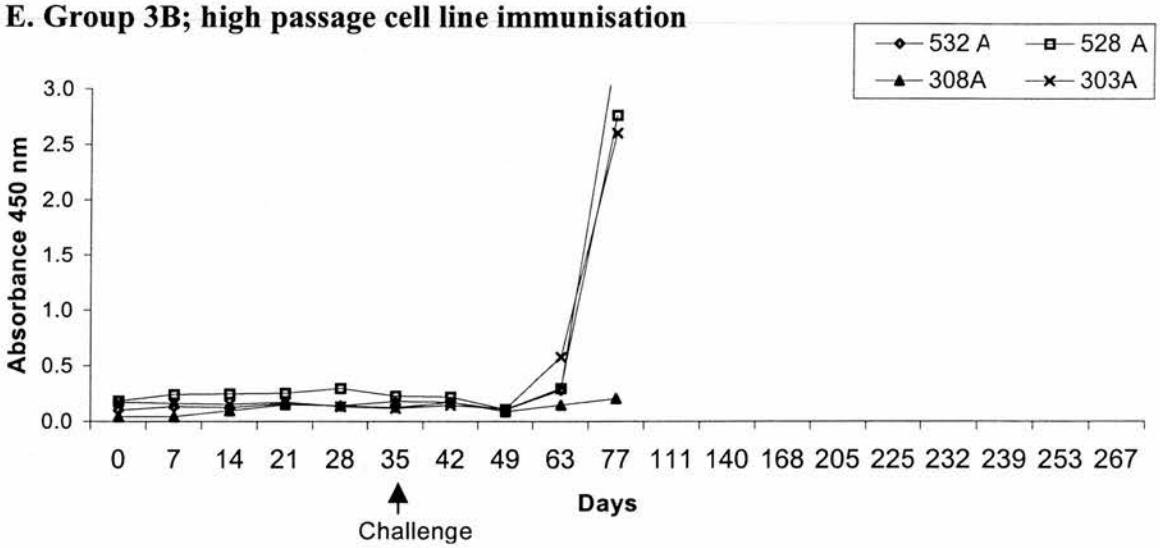
D. Group 2B: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 35 days after primary infection.

E. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 35 days after primary infection.

D. Group 2B; low passage cell line immunisation



E. Group 3B; high passage cell line immunisation



pre-infection OD values by day 56 or 168 in all calves. Following challenge, antibody levels continued to increase (Figure 6.5A).

The IgM levels of calves 17 and 32C in Group 2A increased following immunisation and declined afterwards (Figure 6.5B). Both calves had a peak antibody response following challenge. In calves 22A, 24B and 892A, the antibody response remained low but increased slightly before and after challenge (Figure 6.5B).

The antibody response varied during the course of immunisation and challenge among individual animals in Group 2B (Figure 6.5D). In all calves, antibody levels were detectable by day 14 or 21 after immunisation. Calf 309A showed a low antibody response to immunisation and challenge. Calves 302A, 535A and 15 had two peaks of antibody response, one post-immunisation and another post-challenge.

The antibody profile of calves in Group 3A was similar to that described for the IgG antibodies, but with a lower response after the immunisation. Antibody levels reached twice the pre-infection OD level between days 28 (calves 54C and 891A) and 85 (calves 26A and 32A). There was little or no antibody response after challenge (Figure 6.5C). IgM levels of all calves in Group 3B remained around the pre-immunisation OD values following immunisation, except calf 308A (Figure 6.5E). An increase in antibody level was detected in all calves following challenge.

iii) IgM Tash-2 ELISA

Results of IgM antibody responses of experimentally infected animals in IgM Tash-2 ELISA are shown in Figure 6.6A-E. In general, high pre-infection OD values were detected in comparison to OD values obtained with the IgG Tash-2 ELISA.

Calves in Group 1 had detectable Tash-2 specific IgM response by days 28 (calf 897A) or 84 (calves 21A, 23B, 48C) after primary infection (Figure 6.6A). Antibody levels of calf 21A fluctuated until day 84, and then increased to a very high level by day 112. In all calves, antibody levels remained high until and after challenge.

The IgM response was detected earlier than the IgG response in calves in Group 2A and 2B. The antibody levels started to increase from day 14 onwards,

reached a very high level by day 85 and remained high throughout the infection and challenge. A marked fluctuation in IgM antibody levels occurred (Figure 6.6B and D).

The antibody responses of calves in Group 3A increased from day 56 (calf 54C) or 84 (calves 26A, 32A and 891A). An increase in antibody level was observed in three out of 4 calves following challenge (Figure 6.6C). There was either no difference (calves 532, 528 and 303A) or a slight increase (calf 308A) in IgM levels following immunisation in Group 3B (Figure 6.6E). IgM antibody responses increased quickly and to high OD values following challenge except calf 308A.

6.3.2. IFAT Results of Experimentally Infected Animals

The antibody profiles determined by IFAT of two representative calves from each group are shown in Figures 6.7A-E.

Antibody levels to both macroschizonts and piroplasms rose from negative pre-infection values to positive values between days 28 and 42 in all calves in Group 1 (sporozoite infection). The antibody levels remained positive and at relatively high values with only a slight decrease before challenge. Antibody levels for both antigens increased in all calves after challenge.

Calves in Groups 2A and 2B (low passage cell line infection) showed an earlier response to both antigens than calves infected with sporozoites. All animals within this group became positive between day 14 and 21 and remained positive until and after challenge.

Anti-macroschizont antibodies could be detected in animals immunised with the high passage cell line (Groups 3A and 3B) between days 14 and 21. Antibody levels of all calves in Group 3A declined and became undetectable in two calves by challenge. Seven out of eight calves became positive for anti-piroplasm antibody by day 35. Calf 532 did not give a positive response prior to challenge. Anti-piroplasm antibody levels detected by IFAT were low in these animals and could be detected for only a relatively short period of time compared to calves infected with sporozoites and with the low passage line. Following challenge all calves seroconverted to both antigens. In addition, the antibody response to challenge was more rapid than that obtained following the primary immunisation.

Figure 6.7. Antibody responses of two representative calves from each Group, which were infected with *T. annulata* Ankara sporozoites or immunised with the high or low passage cell lines, detected by both the macroschizont and piroplasm IFAT.

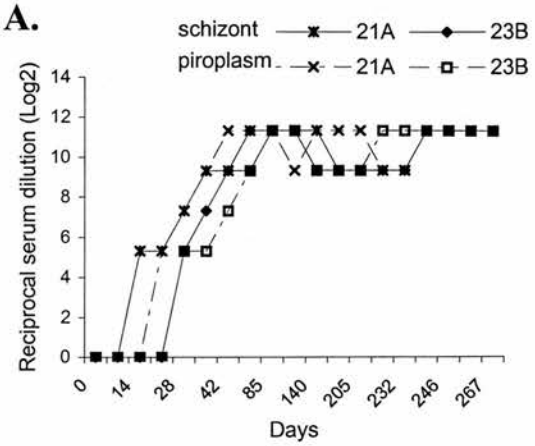
A. Group 1: Calves infected with *T. annulata* Ankara sporozoites and challenged 7 months after primary infection.

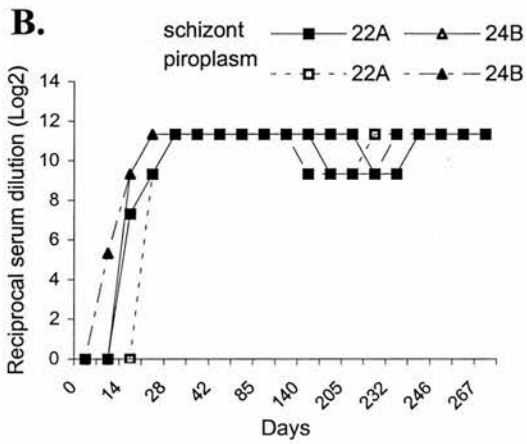
B. Group 2A: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 7 months after primary infection.

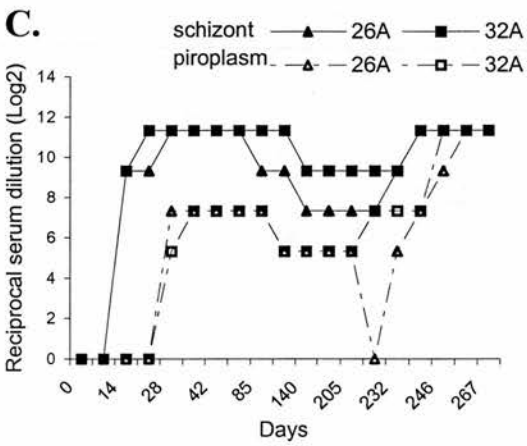
C. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 7 months after primary infection

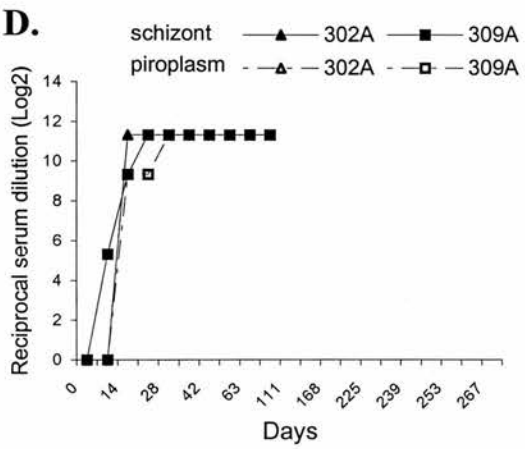
D. Group 2B: Calves immunised with *T. annulata* Ankara low passage cell line and challenged 35 days after primary infection.

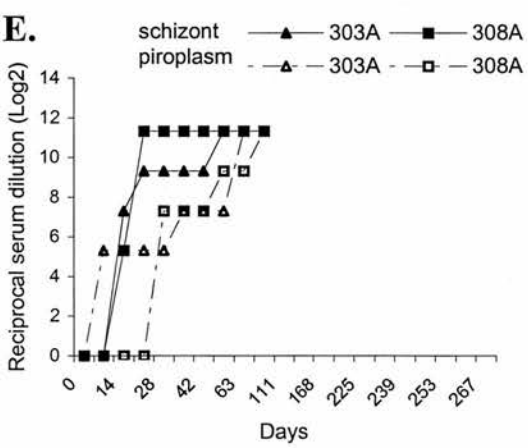
E. Group 3A: Calves immunised with *T. annulata* Ankara/Pendik high passage cell line and challenged 35 days after primary infection.


A.
schizont 21A 23B
piroplasm 21A 23B
Reciprocal serum dilution (Log2)
0 2 4 6 8 10 12 14
0 14 28 42 85 140 205 232 246 267
Days


B.
schizont 22A 24B
piroplasm 22A 24B
Reciprocal serum dilution (Log2)
0 2 4 6 8 10 12 14
0 14 28 42 85 140 205 232 246 267
Days


C.
schizont 26A 32A
piroplasm 26A 32A
Reciprocal serum dilution (Log2)
0 2 4 6 8 10 12 14
0 14 28 42 85 140 205 232 246 267
Days


D.
schizont 302A 309A
piroplasm 302A 309A
Reciprocal serum dilution (Log2)
0 2 4 6 8 10 12 14
0 14 28 42 63 111 168 225 239 253 267
Days


E.
schizont 303A 308A
piroplasm 303A 308A
Reciprocal serum dilution (Log2)
0 2 4 6 8 10 12 14
0 14 28 42 63 111 168 225 239 253 267
Days

6.3.3. Persistence of Serological Responses to Recombinant Antigens

The persistence of antibody responses against the three recombinant antigens (Tamr-1, NC10-Ssp13 and Tash-2) and the antibody response to challenge were analysed using serum samples obtained from animals in Group A and B (see section 6.2.1.2ii).

6.3.3.1. Serum samples from Group A

Table 6.3 shows the results of the IgG ELISAs using serum samples from calves in Group A. Antibody responses were detected in both Sahiwal heifers and in calf 12A using IgG Tamr-1 ELISA. There were no detectable antibody levels specific to Tamr-1 by the end of the first and the third year. On the other hand, antibodies were detected in three out of five cattle by ELISA using Tash-2 or NC10-Ssp13 recombinant antigens 3 years after the primary infection (Table 6.3). In addition, there was an increase in antibody levels in three of the cattle against both macroschizont antigens following challenge.

6.3.3.2. Serum samples from Group B

Results of IgG Tamr-1 and Tash-2 ELISAs using serum samples from Group B are given in Figures 6.8 and 6.9, respectively. Results indicated that all animals developed antibodies specific to Tamr-1 following primary infection (Figure 6.8). However, during the course of infection, antibody levels declined to below or around cut-off values of 14% or 18% positivity in all animals. Antibody response was detected only in a proportion of animals following challenge.

All animals seroconverted in ELISA using Tash-2 recombinant antigen. In general, antibodies against the Tash-2 were detected later than those for the Tamr-1 antigen. Antibody levels in all cattle fluctuated but remained distinctly above twice pre-infection OD values following infection and challenges (Figure 6.9). In contrast to Tamr-1, antibody response increased in all cattle following heterologous or homologous challenges. Serum samples in this group could not be tested with NC10-Ssp13 ELISA because of time constraints.

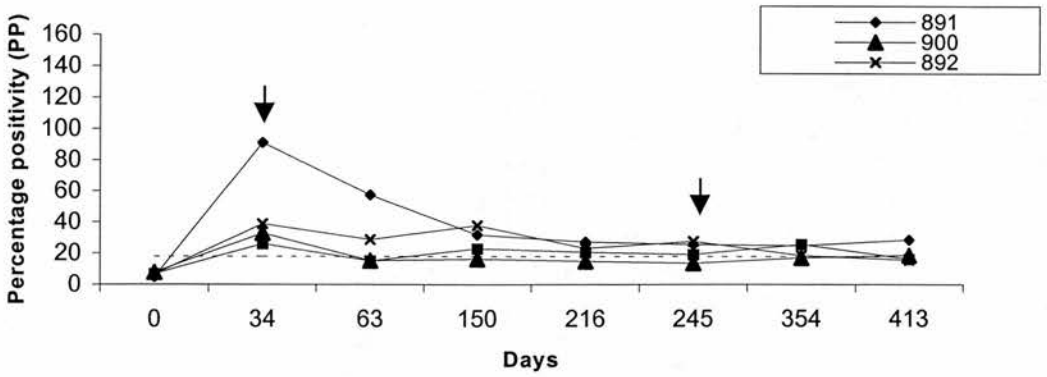
Table 6.3. Persistence of the antibody responses against the Tamr-1, NC10Ssp13 and Tash-2 recombinant antigens in Group A cattle detected by IgG ELISAs. The results are given as PP for Tamr-1 ELISA and OD values for Tash-2 and NC10-Ssp13 ELISAs.

	Tamr-1 / PP[a]					Tash-2/ OD[b]					NC10-Ssp13/OD[b]				
Calf No	488	489	10A	12A	14A	488	489	10A	12A	14A	488	489	10A	12A	14A
Day															
0	2.6	1.6	5.4	6.6	5.0	0.104	0.061	0.192	0.218	0.222	0.137	0.123	0.255	0.502	0.156
28	**18.0**	**19.4**	9.0	**14.7**	4.1	0.069	0.090	0.139	0.103	0.120	**0.409**	**0.626**	0.404	0.583	0.097
57	**14.7**	**22.7**	na	na	na	0.086	**0.200**	na	na	na	**0.328**	**1.104**	na	na	na
84	**15.0**	**22.3**	na	na	na	0.128	**0.502**	na	na	na	**0.286**	**1.047**	na	na	na
365	5.2	7.5	na	na	na	**0.288**	**0.301**	na	na	na	0.236	**0.408**	na	na	na
3 years	3.3	3.1	9.4	8.4	12.4	0.144	0.111	**0.664**	**0.897**	**0.553**	0.216	**0.348**	**0.525**	0.532	**0.463**
Challenge	na	na				na	na				na	na			
28			7.5	7.7	11.8			**1.226**	**1.533**	**1.307**			**1.150**	0.968	**0.600**

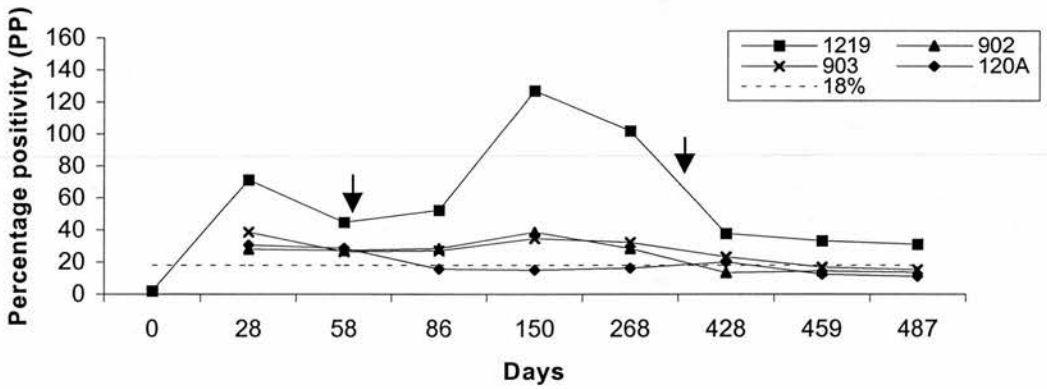
Bold numbers indicate the values above the cut off values (twice the pre-infection OD).
[a]: 14 PP is cut off value for IgG Tamr-1 ELISA.
[b]: Twice the pre-infection OD value of each calf is cut-off value
na. not available

Figure 6.8. Persistence of antibody response against the recombinant antigen Tamr-1 in Group B cattle infected with *T. annulata* sporozoite stabilates detected by ELISA. Calves challenged once or several times with homologous or heterologous *T. annulata* sporozoite stabilate. Arrows shows day of challenges. (A) Calves 891, 892, 899 and 900; (B) calves 903, 902 120A and 1219; (C) calves 1052 and 1055; (D) 11434. Infection and challenge(s) of these calves were carried out as described in Table 6.2.

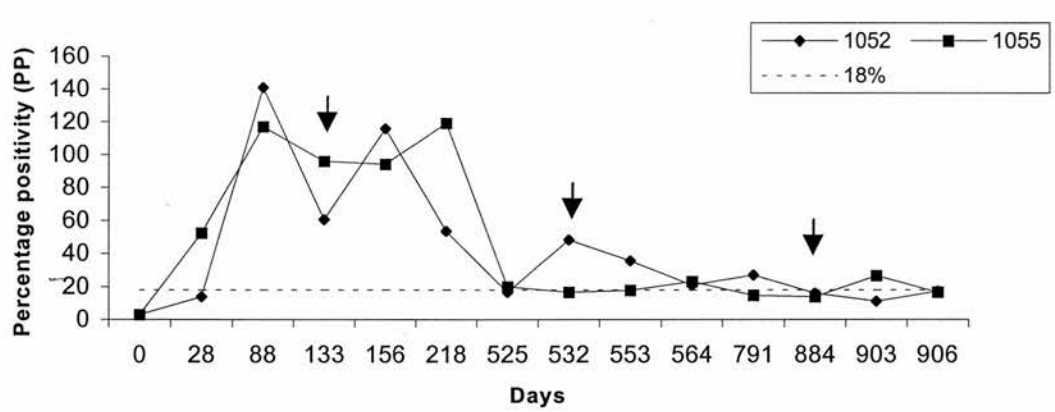
A)


Percentage positivity (PP)
160
140
120
100
80
60
40
20
0
0
34
63
150
216
245
354
413
Days
891
900
892

B)


Percentage positivity (PP)
160
140
120
100
80
60
40
20
0
0
28
58
86
150
268
428
459
487
Days
1219
902
903
120A
18%

C)


Percentage positivity (PP)
160
140
120
100
80
60
40
20
0
0
28
88
133
156
218
525
532
553
564
791
884
903
906
Days
1052
1055
18%

D)

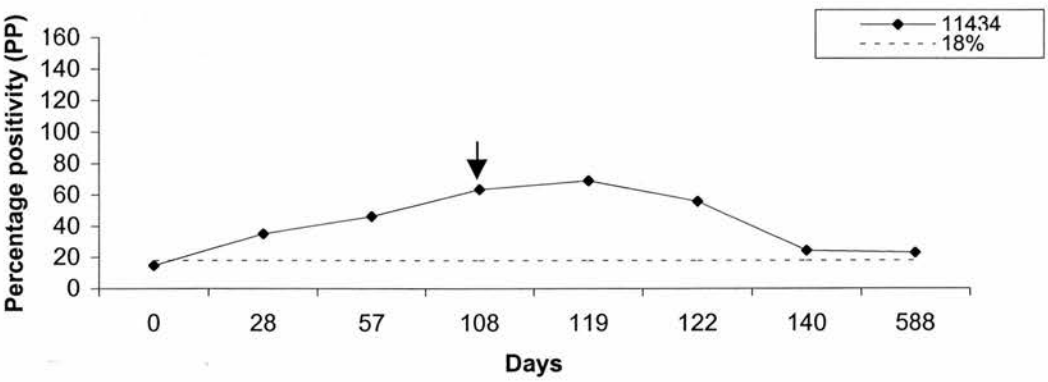
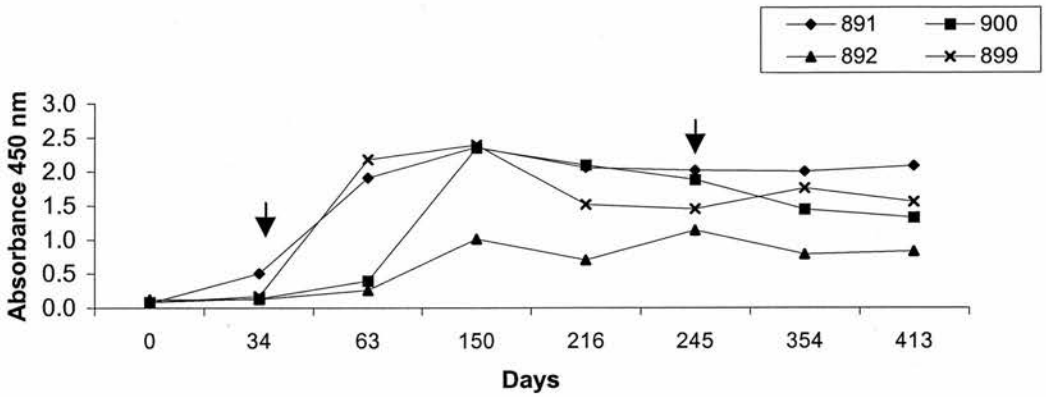
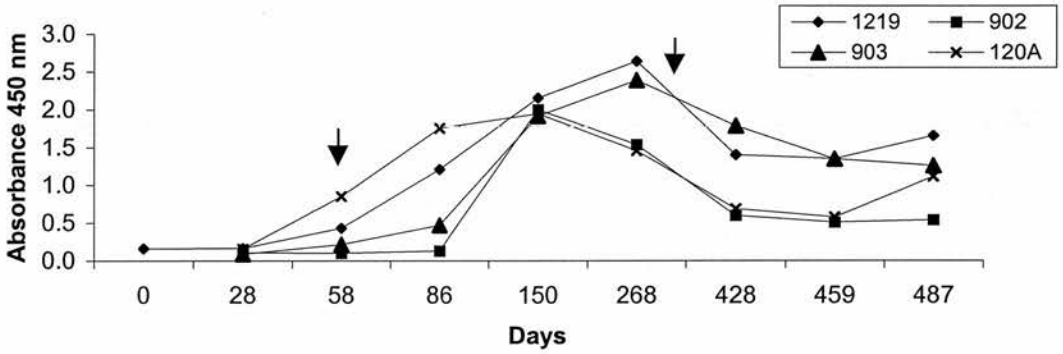

Percentage positivity (PP)
160
140
120
100
80
60
40
20
0
0
28
57
108
119
122
140
588
Days
11434
18%

Figure 6.9. Persistence of antibody response against the recombinant antigen Tash-2 in Group B cattle infected with *T. annulata* sporozoite stabilates detected by ELISA. Calves challenged once or several times with homologous or heterologous *T. annulata* sporozoite stabilate. Arrows shows day of challenges. (A) Calves 891, 892, 899 and 900; (B) calves 903, 902 120A and 1219; (C) calves 1052 and 1055; (D) 11434. Infection and challenge(s) of these calves were carried out as described in Table 6.2.

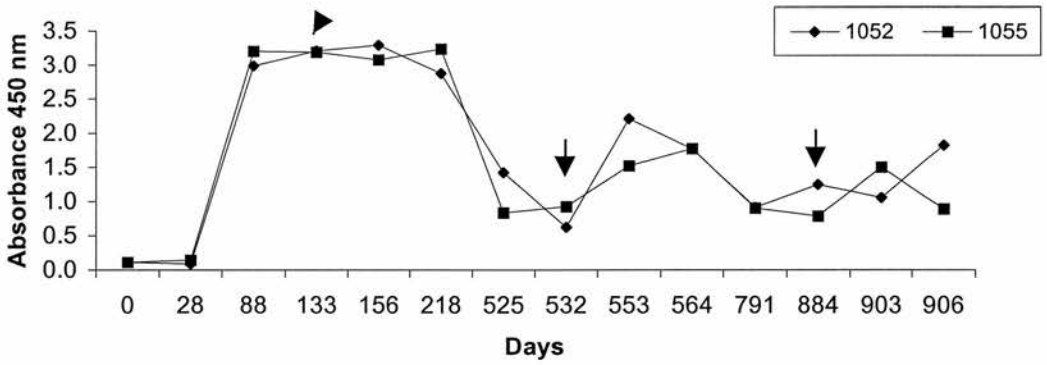
A)


891
900
892
899
Absorbance 450 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
34
63
150
216
245
354
413
Days

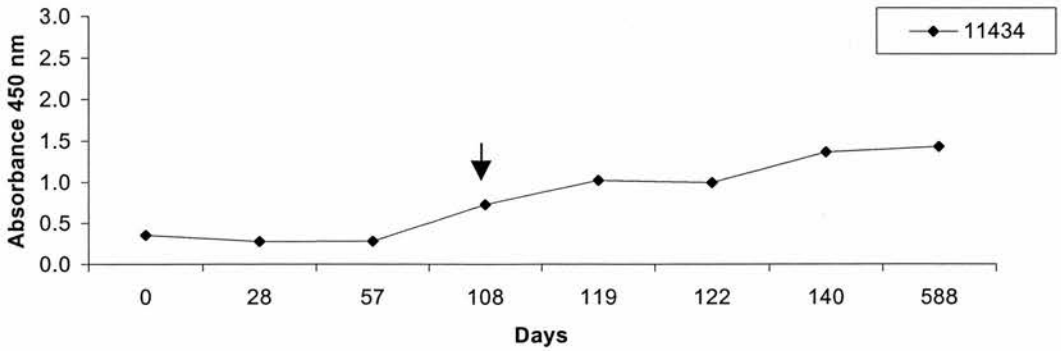
B)


1219
902
903
120A
Absorbance 450 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
28
58
86
150
268
428
459
487
Days

C)


1052
1055
Absorbance 450 nm
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
28
88
133
156
218
525
532
553
564
791
884
903
906
Days

D)


11434
Absorbance 450 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
28
57
108
119
122
140
588
Days

6.3.4. Field Serum Samples

Point prevalence values of *T. annulata* infection in cattle around Aydin province of Turkey which were obtained using the IgG Tamr-1 ELISA and both the piroplasm and macroschizont IFAT are given in Table 6.4. The presence of piroplasm parasitaemia and PCR results of these animals are also shown in Table 6.4. Numbers of cattle found to be positive by the IgG Tamr-1 ELISA, macroschizont IFAT and PCR were similar at 14 PP cut-off value. However, they did not detect antibodies in the same animals. For example, nine cattle found to be negative by Tamr-1 ELISA were positive by the macroschizont IFAT and the 12 Tamr-1 positive cattle were negative by the macroschizont IFAT. The Tamr-1 ELISA detected significantly more animals as positive than the piroplasm IFAT ($p = 0.046$) and the examination of Giemsa's stained blood smears ($p = 0.013$). The number of animals detected by Tamr-1 ELISA was significantly less at 18 PP cut-off value than at 14 PP cut-off value ($p = 0.001$), IFAT macroschizont ($p = 0.001$) and PCR ($p = 0.02$).

If the PCR results are accepted as 'gold standard', the number of false-positive animals detected by the IgG Tamr-1 ELISA at 14PP cut-off value (n=12) and the macroschizont IFAT (n=8) was close to each other. Similarly, the difference between the number of false-positive animals detected by the IgG Tamr-1 ELISA at 18 PP cut-off value (n= 6) and the piroplasm IFAT (n= 4) was small.

6.4. DISCUSSION

In this study, the stage-specific recombinant antigens, Tamr-1, NC10-Ssp13 and Tash-2, were screened against antisera produced to determine whether the Tamr-1 ELISA could distinguish vaccinated animals from those naturally infected animals when it is used together with either the NC10-Ssp13 or Tash-2 ELISAs.

Cut-off values for IgG Tamr-1 ELISA were determined as 14 and 18 PP as described in Chapter 5. The diagnostic sensitivity and specificity of the NC10-Ssp13 and Tash-2 ELISAs and cut-off values could not be determined because of time constraints. In order to assess the increase in antibody levels of individual calves following infection the cut-off values for the IgM Tamr-1 ELISA and the IgG and IgM ELISAs of NC10-Ssp13 and Tash-2 were used as twice the absorbance value of pre-infection serum for each calf (de Savigny and Voller, 1980).

Table 6.4. Prevalence values of *T. annulata* infection in cattle obtained using Giemsa stained blood smears, PCR, IFAT piroplasm and macroschizont antigens and the IgG Tamr-1 ELISA, with associated 95% confidence intervals (CI) in brackets.

Number of animals	**Blood smear**	**PCR**	**IFAT**		**IgG Tamr-1 ELISA**	
			Macroschizont	Piroplasm	14 PP cut-off	18 PP cut off
79	63.3%	73.4%	79.8% (70.9-88.7)	67.1% (56.7-77.5)	81.0% (72.3-89.7)	55.7% (44.7-66.7)

In most of the animals that were infected with sporozoites (Group 1), antibody responses to Tamr-1 antigen were detected relatively late, i.e. between days 28 and 56, in comparison to IFAT using the macroschizont and piroplasm antigens (days 28 and 35). Furthermore, calves in Group 1 had lower PP values than those infected with the low passage cell line (Group 2A and 2B). This relatively late and low antibody response might be due to the treatment of animals in Group 1 with buparvaquone earlier than those in Group 2A and 2B. Calves in Group 1 were treated with buparvaquone 4 or 5 days after the detection of piroplasms in the blood smears while calves in Group 2A and 2B exhibited piroplasms for 13 to 15 days before treatment. This would in effect reduce the time during which the immune system is exposed to piroplasms in Group 1, leading to a delay in the production of antibodies against the Tamr-1 antigen. The early antibody response observed in animals infected with the low passage cell line (Group 2A and 2B) might also be due to the appearance of piroplasms in circulation as early as day 7 in some of the calves in contrast to at day 9 in animals infected with sporozoite infection. The early detection of piroplasms in animals infected with the low passage cell line could be due to fact that these macroschizont infected cells start differentiating to merozoites soon after inoculation.

As expected, no antibody responses were detected in calves immunised with the high passage cell line (Group 3A and 3B) using the Tamr-1 ELISA. The antibody responses of these animals obtained with the Tamr-1 ELISA were below 14 and 18 PP indicating that this antigen could be used to distinguish vaccinated animals from naturally infected or challenged animals. No piroplasms were observed in calves in these groups. It would appear from these observations that macroschizonts from high passage cell lines did not differentiate to merozoites *in vivo* as observed *in vitro* (Ilhan, 1995), or, if such differentiation occurred, these merozoites did not develop to produce piroplasms and to stimulate antibodies detectable by the ELISA using Tamr-1 antigen.

Antibodies against NC10-Ssp13 and Tash-2 antigens could be detected by IgG ELISAs in animals either infected with sporozoites (Group 1) or immunised with the low passage cell line (Group 2A and 2B). Antibody responses were also detected in calves immunised with the high passage cell line (Group 3A). Although

the antibody levels were around twice the pre-infection values this is not clearly demonstrated in Figures 6.2 and 6.3. This is simply due to fact that the scale of figures was adjusted according to antibody levels of animals in Group 1 and 2. Antibodies against NC10-Ssp13 and Tash-2 were detected later (day 84) than antibodies against Tamr-1 ELISA (day 35) in Group 1. Similarly, anti-Tash-2 antibodies were detected later than anti-NC10-Ssp13 and anti-Tamr-1 antibodies in Group 2A and 2B. This relatively late appearance of the antibodies against NC10-Ssp13 and Tash-2 correlates with some animals being negative in Western blot analysis as described in Chapter 5. Most of the serum samples, which did not react, with NC10-Ssp13 and Tash-2 in Western blotting, were obtained at day 28 following infection.

The OD values obtained by IgG Tash-2 ELISA in calves infected with sporozoites were close to pre-infection values for the first three months following infection and reached very high levels afterwards. It is well established that antibody levels increase with time after immunisation associated with a progressive change in the quality of antibody (Eisen and Siskind, 1964). Specifically, low affinity antibodies are produced soon after infection, whereas high affinity antibodies are produced at later stages of infection. This is referred to as 'affinity maturation' (Roitt, 1994). Previous observations indicated that the low antigen concentrations and high serum dilutions favour the detection of high affinity antibodies (Lew, 1984). This could be a reason for not detecting antibodies during the first three months of infection by the IgG Tash-2 ELISA. Since the IgG Tash-2 ELISA was established using high serum dilution (1:400) and low antigen concentration (0.08μg/ml), it is most likely that the Tash-2 ELISA detected high affinity antibodies. This problem could be eliminated by increasing epitope density, i.e. increasing antigen concentration to preferentially improve the binding of lower affinity antibodies (Butler, 1988). In addition, low affinity antibodies might not be detected due to dissociation from the solid phase antigen during washing steps (Devey and Steward, 1988).

No antibody response was observed in calves immunised with the high passage cell line (Group 3A and 3B) by day 111 and antibody levels only reached twice the pre-infection values by the time of challenge. These low level antibody

responses could be due to the presence of low affinity antibodies. Alternatively, the protein encoded by the Tash-2 gene may not be expressed in the high passage cell line used in the current study (D. Swan, pers. comm.).

The IgM response detected by IgM Tamr-1 ELISA occurred earlier than the IgG response in calves infected with either sporozoites (Group 1) or the low passage cell line (Group 2A and 2B). The response declined by day 84 after infection or immunisation, indicating a classical IgM response. Thereafter, IgM levels fluctuated during the course of infection and, in some cases, remained above twice the pre-infection levels until challenge. In contrast to the IgM response to Tamr-1, IgM responses against NC10-Ssp13 and Tash-2 were similar to IgG responses to the same antigens. IgM levels for NC10-Ssp13 and Tash-2 fluctuated but remained above the cut-off values until challenge in most of calves. This is in contrast to previous observations which indicated that IgM levels increased from day 21 and declined by day 40 following infection with *T. parva* (Spooner *et al.*, 1973). On the other hand, it was shown that high levels of IgM response could be maintained for more than one year in some trypanosome-infected cattle (Luckins and Mehlitz, 1976). High and continuous IgM antibody responses following the primary infection could be due to the development of a carrier state of the parasite. This would result in continuous stimulation of antibody production. Since the NC10-Ssp13 antigen is expressed mainly in the macroschizont stage of the parasite and Tash-2 antigen in both the macroschizont and piroplasm stages, continuous IgM antibody responses against these two antigens at later stages of infection would indicate that calves infected with sporozoites or macroschizont infected cell lines are carrier for both macroschizonts and piroplasms.

Results obtained from ELISAs performed with three recombinant antigens were compared with the conventional IFAT using both macroschizont and piroplasm antigens. The IFAT using the macroschizont antigen is currently used as the standard serological assay for *T. annulata* to detect both vaccinated and naturally infected animals (Pipano, 1974; Anon, 1997). All calves used in the current study sero-converted with IFAT using both macroschizont and piroplasm antigens after infection or immunisation. Antibodies were detected earlier with the IFAT than with any of the IgG or IgM ELISAs in calves infected with sporozoites or immunised with

the low and high passage cell lines. In general, piroplasm antibodies in IFAT were detected later than macroschizont antibodies fitting in with the normal life cycle sequence of the parasite. This is probably because the merozoite/piroplasm stage follows the macroschizont stage in the *Theileria* vertebrate life cycle. However, animals infected with the low passage cell line developed antibodies against piroplasms and macroschizonts simultaneously. The antibody response to piroplasms was observed earlier in groups of animals that were infected with the low passage cell line than in animals that were infected with sporozoites. This is explained by the early appearance of piroplasms in the blood (day 7) in some calves infected with the low passage cell line (Chapter 3). This correlates with the apparently anomalous development of merozoites and piroplasms from inoculated macroschizont infected cells before their transfer to host cells. In contrast, piroplasms did not appear before day 9 in sporozoite infected animals (Chapter 3).

Anti-piroplasm antibodies could be detected in animals immunised with the high passage cell line even though piroplasms were not seen. However, the antibody levels and duration of the responses were lower and shorter than those observed after infection with the low passage cell line. Although piroplasms were not observed in Giemsa's stained blood smears, detection of anti-piroplasm antibody by IFAT would be expected due to the presence of macroschizont antigens common to piroplasms (Bell-Sakyi and Williamson, 1994; Knight *et al.*, 1998).

The IgG Tamr-1 ELISA detected antibodies for over 7 months following the primary infection and over a year after challenge. The challenge only slightly affected the antibody levels against the Tamr-1 antigen. On the other hand, both the IgG NC10-Ssp13 and Tash-2 ELISAs detected antibodies for three years even in absence of challenge. All these animals 'naturally' infected with sporozoites were piroplasm carriers for three years, implying the persistent presence of macroschizonts and their differentiation to merozoites/ piroplasms. However, it should be noted that no age-matched control serum samples were available for these experiments. Instead, pre-infection serum samples were used as negative reference. This is important since antibody levels change with age and increase with bacterial or viral infections. This may, in turn, enhance non-specific binding (Venkatesan and Wakelin, 1993). For a

more comprehensive study, therefore, it would be useful to repeat these experiments after establishing appropriate cut-off values.

The current study demonstrates that, in contrast to the Tamr-1, the antibody response to the Tash-2 antigen increases following challenge. Data obtained from this experimental study using serum samples to test the persistence of antibody responses indicate that immune responses against the macroschizont stage was highly effective and blocked any significance recrudescence of piroplasms. This suggests that challenges did not stimulate an antibody response against piroplasms, but against macroschizonts.

The antibody response of cattle with a history of theileriosis in Aydin province of Turkey was examined in the current study by the IgG Tamr-1 ELISA and results were compared with the following diagnostic tests: the macroschizont and piroplasm IFAT, Giemsa's stained blood smears and PCR. The results obtained from these studies indicated that the IgG Tamr-1 ELISA is as sensitive as the macroschizont IFAT and more sensitive than the piroplasm IFAT at 14 PP cut off value. The IgG Tamr-1 ELISA was also as sensitive as the piroplasm IFAT at 18 PP cut off value. These results suggest that the IgG Tamr-1 ELISA could be as powerful a diagnostic tool as the macroschizont and piroplasm IFAT for use in epidemiological studies of theileriosis. However, the diagnostic value of the Tamr-1 ELISA in the field can only be assessed using a larger number of field samples that can truly represent the actual field situation.

The present study also indicated that the macroschizont IFAT is more sensitive than the piroplasm IFAT. A similar observation was previously made by (Darghouth *et al.*, 1996b). This is expected since serum samples examined in the current study were obtained during disease season (June-July). As discussed above, the antibody response to macroschizonts occurs more rapid than the antibody response to piroplasms in animals newly exposed to theileriosis. These results indicate that timing of sample collection should be considered in evaluating the sensitivity of IFAT.

The aim of the current study was to distinguish between vaccinated and naturally infected animals by combining results of Tamr-1 ELISA with either NC10-Ssp13 or Tash-2 ELISAs. According to this scheme, if both Tamr-1 ELISA and

NC10-Ssp13 / Tash-2 are positive, it can be concluded that the animal has been infected or challenged with sporozoites. On the other hand, a negative result with the Tamr-1 ELISA and a positive result with NC10-Ssp13 / Tash-2 would indicate that the animal was only immunised with an attenuated macroschizont infected cell line, and not yet exposed to field challenge with tick transmitted sporozoites.

The results of this study show that, ELISAs using the Tamr-1, NC10-Ssp13 or Tash-2 recombinant antigens cannot be used to distinguish animals vaccinated with the attenuated high passage cell line from those that are naturally infected. Because the sensitivity of the Tamr-1 is insufficient (see Chapter 5) and neither the NC10-Ssp13 ELISA nor the Tash-2 ELISA detect sensitively antibodies in animals immunised with high passage cell line. Both IgG Tamr-1 and Tash-2 ELISAs, however, could serve as powerful diagnostic tools in epidemiological studies of theileriosis, as ELISA systems permit processing of larger numbers of samples than IFAT and give objective results. Nevertheless, it should be noted that the sensitivity of the Tash-2 ELISA was lower during the first three months following infection with sporozoites. Therefore, antibody responses to the Tash-2 antigen should ideally be examined three months after the disease season.

In the next chapter, the detection of the carrier-state of *T. annulata* in cattle using a nested PCR will be described. The nested PCR distinguishes *T. annulata* from *T. buffeli* in cattle and *T. annulata* from *T. lestoquardi and B. equi* in vector ticks. Such sensitive and specific tests would not only complement the sero-epidemiological studies of theileriosis but also help targeting the limited amount of vaccine produced each year to those areas most in need.

CHAPTER SEVEN

DETECTION OF *THEILERIA ANNULATA* IN CATTLE BY PCR USING THE TAMS-1 GENE SEQUENCES

7.1. INTRODUCTION

The purpose of this study was to develop a sensitive and specific diagnostic test for the detection of the carrier state of tropical theileriosis. To this end, a nested PCR using primers derived from the gene encoding the 30 kDa major merozoite surface antigen (Tams-1) of *T. annulata* was established.

Animals that are exposed to sporozoites for the first time become infected and the outcome may be sub-clinical infection or clinical disease leading to death or survival (Sergent *et al.*, 1945; Neitz, 1957). In many *Theileria* infected animals, infection persists after recovery and is maintained by two stages of the parasite's life cycle: macroschizont and piroplasm (Sergent *et al.*, 1940; Neitz, 1957; Sergent, 1963). The carrier state of *Theileria* spp is believed to be maintained by the persistent and slow division of macroschizonts that give rise to piroplasms (Norval *et al.*, 1992). The division and long-term persistence of *T. annulata* piroplasms within erythrocytes have been demonstrated (Conrad, 1983; Conrad *et al.*, 1985). However, there is no experimental evidence indicating that piroplasms re-invade host erythrocytes following multiplication.

Vector ticks, which feed on carrier cattle and become infected, have the potential to transmit the parasite to a susceptible host. Therefore, the carrier state of *T. annulata* is of great importance in the epidemiology of the disease. The detection of carrier animals is needed not only for a better understanding of the epidemiology of theileriosis but also for effective implementation of control programmes. The detection of the carrier state of the parasite could also indicate the immune status of animals in the field since animals that survive the initial infection with sporozoites are fully protected against homologous and heterologous sporozoite challenge (Barnett, 1963; Ilhan, 1995; Chapter 3).

Traditionally, the carrier-state of theileriosis has been determined by microscopic examination of Giemsa's stained blood smears for the presence of piroplasms. This method, which requires considerable time and expertise, is not sensitive since piroplasm parasitaemia is often too low to be detected in many carriers (Norval *et al.*, 1992). This results in underestimation of the true prevalence of the carrier state in a population. Another drawback of microscopic examination for the detection of the carrier state relates to its specificity. Different piroplasm forms, i.e. rod, oval, comma and rings, predominate in different *Theileria* species (Uilenberg, 1981b). Yet, this can vary during the course of infection (Norval *et al.*, 1992). Therefore, the detection of the carrier state with microscopy can be ambiguous where the distributions of different *Theileria* species overlaps.

Another method that can be used to detect the carrier state of theileriosis is xenodiagnosis. Xenodiagnosis involves the application of vector ticks to test animals, followed by transfer and transtadial transmission to susceptible hosts (Sergent *et al.*, 1945). It is sensitive but impractical and expensive.

As described above, the carrier state of theileriosis is maintained by the macroschizont stage of the parasite. Cell culture methods, which allow the detection of macroschizonts (Brown, 1987), could be used to examine the carrier state of theileriosis. Previous studies demonstrated that macroschizont-infected cell lines can successfully be isolated from animals undergoing clinical disease (Ilhan *et al.*, 1998; Chapter 3) and during recovery and convalescence (Sharma and Brown, 1981). However, macroschizont infected cell lines could not be isolated 56 days after primary infection with sporozoites (Ilhan *et al.*, 1998; Chapter 3), limiting the diagnostic value of this technique for the detection of carrier animals.

It is evident from the above discussion that none of the techniques mentioned above can be relied on individually to reveal the true prevalence of theileriosis. The development of molecular biology has made available tools to detect and identify microorganisms. The PCR which is a method for enzymatic amplification of specific DNA sequences (Mullis and Faloona, 1987; Saiki *et al.*, 1988b) is the most frequently used tool. A PCR-based methodology would offer important advantages over conventional techniques such as greater sensitivity and specificity. The diagnostic value of PCR has been demonstrated in a number of studies on a wide

range of parasites (Figueroa and Buening, 1995; Comes *et al.*, 1996) including *T. annulata* (Ilhan, 1995; d'Oliveira *et al.*, 1995; 1997b).

Preliminary experiments aimed to detect the carrier state of the parasite through PCR utilizing primers from small subunit ribosomal RNA (ssu RNA) (Ilhan, 1995) have failed due to lack of specificity (data not shown). The purpose of the current study was to develop a sensitive PCR, specific to *T. annulata,* with a particular emphasis on the carrier state of the parasite. The PCR described in this chapter utilises primers complementary to sequences in the *Theileria* spp. major merozoite surface antigen gene (Shiels *et al.*, 1995; Katzer *et al.*, 1998b).

7.2. MATERIALS AND METHODS

7.2.1. Parasite Material

7.2.1.1. T. annulata *piroplasm-infected blood*

Sporozoite stabilates of three stocks of *T. annulata*, Ankara (Turkey) (Schein *et al.*, 1975), Gharb (Morocco) (Ouhelli, 1985) and Hisar (India) (Gill *et al.*, 1980), were thawed and used to infect three cattle. When piroplasm parasitaemia was 1 – 5%, blood was taken into EDTA vacutainer tubes and then serially diluted with uninfected bovine blood taken from a different animal to give ten-fold dilutions of piroplasm material from 1% to 10^{-9}%. Care was taken not to use blood from high parasitaemia (5>) when red blood cells can be infected with several piroplasms. Blood-samples were used for DNA isolation immediately or 6 months after storage at –20°C.

7.2.1.2. T. annulata *infected cell lines*

Peripheral blood mononuclear cells (PBM) were isolated from animals diagnosed with tropical theileriosis in Aydin province, western Turkey and were cultured to provide 25 immortal, macroschizont-infected cell lines (see section 7.2.1.4). In addition, previously isolated and cryopreserved cell lines from Morocco (Gharb), Tunisia (Battan) (Ben Miled, 1993), Spain (Caceres) (de Kok *et al.*, 1993), Sudan (Soba) (Melrose *et al.*, 1984), Turkey (Ankara), Israel (Tova) (Pipano *et al.*,

1974), Iran (Razi) (Hooshmand-Rad and Hashemi-Fesharki, 1968) and India (Hisar) were resuscitated and grown as described in section 3.2.4.

7.2.1.3. Other parasites

DNA from several other tick-borne pathogens was used to test for cross-reactivity with *T. annulata* primers. Three *T. lestoquardi*-infected cell lines from Iran, namely Lahr, Shiraz and Kemalabad (Hooshmand-Rad *et al.*, 1993), were used in the current study. *T. buffeli* (Australia) was provided as a blood stabilate from the Tick Fever Research Centre, Wacol, Queensland, Australia. A splenectomised calf was infected with this stabilate. Piroplasm infected blood at a piroplasm parasitaemia of 1% was collected into EDTA vacutainers and stored at - 20°C until DNA was extracted. Four stocks of *Babesia equi,* Kwa Zulu (South Africa) (Zweygarth, pers. comm.), Onderstepoort (South Africa) (Phipps, pers. comm.) USDA/Florida stock (U.S.A.) (Knowles *et al.*, 1991), and "North Africa" (Gerstenberg *et al.*, 1998) were supplied in the form of fresh blood, blood stabilate in DMSO or as isolated merozoites. *B. bigemina* (Muguga, Kenya), *B. bovis* (Nigeria) and *B. divergens* (Stormont, Ireland), kept at CTVM as blood stabilates in DMSO were thawed and the blood used directly to extract DNA.

7.2.1.4. Uninfected samples

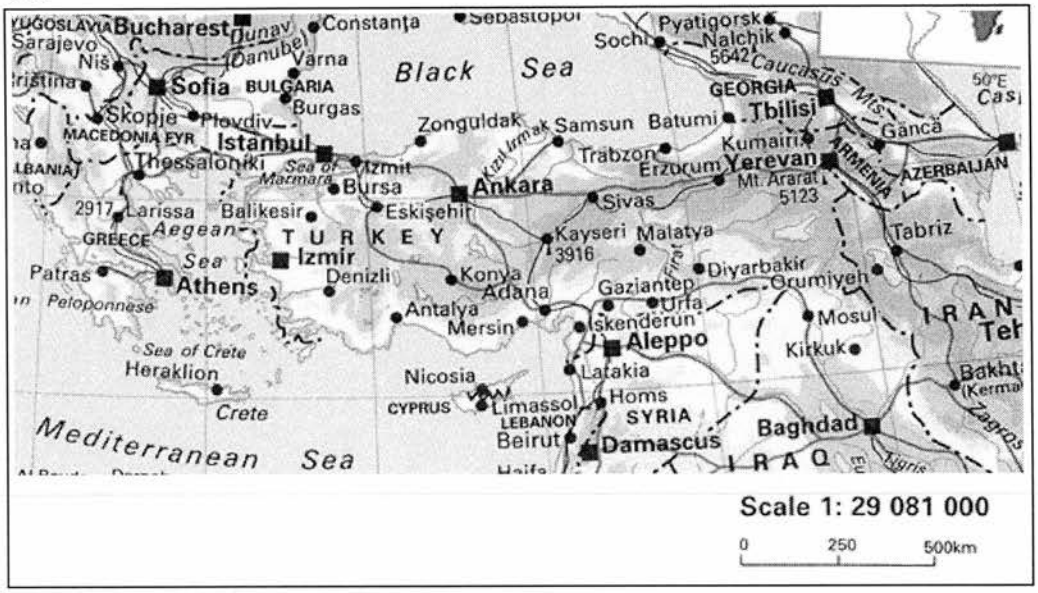
Uninfected bovine blood from naïve Scottish calves from East Lothian was collected into lithium-heparin containing vacutainers and DNA was prepared from 200 µl blood using a Qiagen blood kit or 1 ml blood using saponin lysis buffer. In addition, the PBM isolated from blood according to standard procedures using Ficoll-Paque (Brown, 1983) and DNA was prepared using a Qiagen blood kit.

7.2.1.5. Field samples

In June-July 1996, a pilot study was carried out in 17 districts of Aydin province (Figure 7.1) to assess the suitability of the PCR for field samples. Over a period of five weeks 151 blood samples were collected in EDTA vacutainer tubes from local exotic cattle which had a history of *T. annulata* infection. Each blood sample was used to prepare thin blood smears and also for DNA extraction for PCR

Figure 7.1. Maps of Turkey (A) and Aydin Province (B).

A.


Bucharest
Constanta
Sevastopol
Pyatigorsk
Sochi
Nalchik
5642
Caucasus Mts.
Black Sea
Sofia
BULGARIA
Varna
Burgas
GEORGIA
Tbilisi
Skopje
Plovdiv
MACEDONIA FYR
Istanbul
Zonguldak
Samsun
Batumi
Kumairi
Ganca
Thessaloniki
Trabzon
Erzurum
Yerevan
ARMENIA
AZERBAIJAN
Sea of Marmara
Izmit
Bursa
Ankara
Mt. Ararat
5123
2917
Larissa
Balikesir
Eskişehir
Sivas
Aegean Sea
GREECE
TURKEY
Kayseri
Malatya
3916
Tabriz
Izmir
Patras
Athens
Denizli
Konya
Adana
Diyarbakir
Gaziantep
Urmiyeh
Peloponnese
Antalya
Mersin
Iskenderun
Urfa
Mosul
IRAN
Aleppo
Kirkuk
Sea of Crete
Heraklion
Nicosia
Latakia
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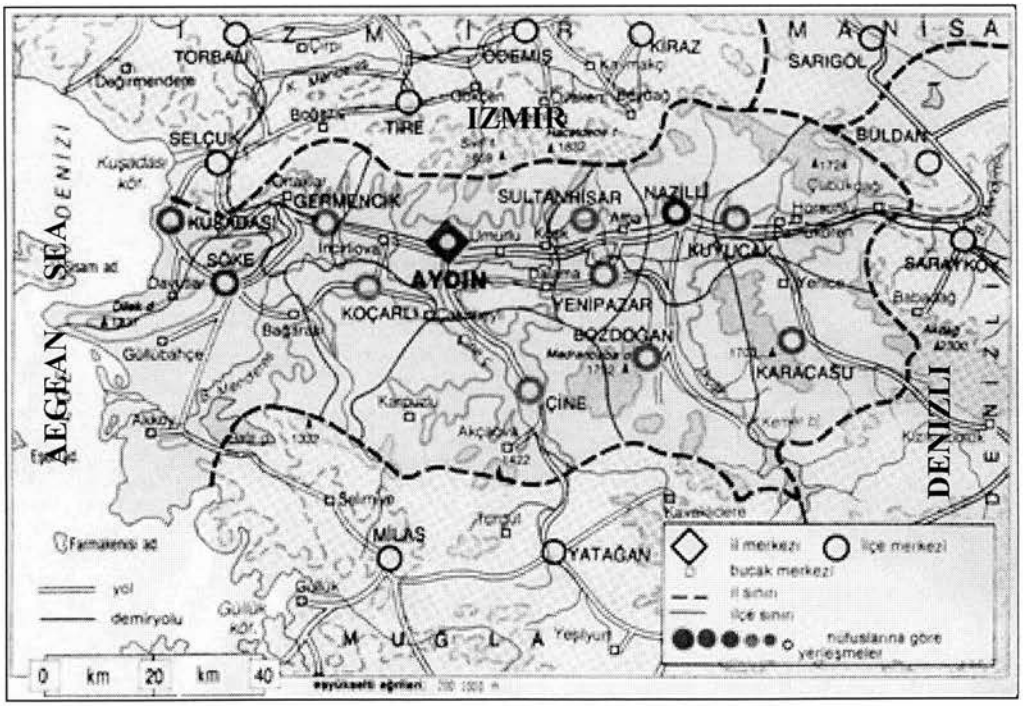
B.


İZMİR
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SARIGÖL
Değirmendere
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BULDAN
Kuşadası Körfezi
GERMENCİK
SULTANHİSAR
NAZİLLİ
KUŞADASI
İncirliova
KUYUCAK
SARAYKÖY
SÖKE
AYDIN
Davutlar
YENİPAZAR
KOÇARLI
BOZDOĞAN
Bağarası
KARACASU
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AEGEAN SEA
DENİZLİ
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analysis. Blood smears were fixed in methanol, stained with Giemsa's and examined for the presence of blood protozoa at 1,000 x magnification under high power light microscopy. The degree of parasitaemia was recorded as the percentage of infected red blood cells after counting 1,000 red blood cells. If the parasitaemia was less than 0.1%, the number of piroplasms per 50 fields was assessed. The smear was recorded as negative if no piroplasms were observed in 50 fields i.e. approximately 25,000 red blood cells.

For DNA isolation, a 1ml sample of EDTA blood was aliquoted and kept at –20°C until use. The DNA was extracted following saponin lysis of the blood as described in section 7.2.2 and DNA samples stored for up to one year at –20°C.

Biopsies taken from enlarged lymph nodes of 39 cattle showing acute theileriosis symptoms were Giemsa's stained and examined for the presence macroschizonts.

Jugular blood was taken into EDTA vacutainers in order to isolate *T. annulata* macroschizont infected cells from 67 cattle showing patent infection either before or after treatment for theileriosis with buparvaquone. Blood was processed either the same day or the following day as described by Brown (1983). Briefly, blood was centrifuged at 500 x g for 10 minutes and the plasma was discarded. The buffy coat was removed and placed into a 25 cm^2 tissue culture flask containing 4.5 ml cold, RPMI-1640 (Gibco) medium supplemented with 20% heat inactivated Serum Supreme (Bio-Whittaker), 2mM L-Glutamine, 150 μg/ml streptomycin, 150 iu/ml penicillin and 5 x 10^{-5} M 2-mercapto-ethanol. The flask was placed vertically in an incubator at 37°C with the cap tight. The medium was changed twice a week by taking 3 ml of medium without disturbing the cells and replacing it with the same volume of fresh warm medium. Culture smears were prepared weekly to assess whether macroschizont infected cells were established. To do this, 0.5 ml of culture suspension was centrifuged at 300 x g for 10 minutes. A thick smear was prepared from the pellet and Giemsa's stained. When approximately 50% or more cells in the smear were infected 2.5 ml of culture suspension was transferred into a new flask containing 7.5 ml of fresh media and placed horizontally in an incubator.

7.2.2. DNA Extraction

7.2.2.1. Preparation of DNA from cell cultures

Standard phenol-chloroform extraction was used to extract DNA from macroschizont-infected cell lines and uninfected bovine PBM as described previously (Sambrook *et al.*, 1989) (see section 4.2.18.1).

7.2.2.2. Preparation of DNA from blood

DNA from blood samples was extracted either using QIAamp Blood Kit (Qiagen Ltd., UK) or the saponin lysis method (Barker *et al.*, 1992). The extraction of DNA from both uninfected and infected blood using the QIAamp Blood Kit was performed according to the manufacturer's instructions. DNA was extracted from 200 µl of blood. Blood samples were mixed with 25 µl Qiagen protease and 200 µl Buffer AL by vortexing for 15 seconds. The mixture was incubated at 70°C for 10 minutes. The samples were placed in QIAamp spin columns and centrifuged at 6,000 x g for 1 min. The extracted DNA was washed twice with 500 µl of AW buffer (Qiagen) by centrifugation of columns for 1 minute at 6,000 x g. An extra spin at 13,000 x g was performed to remove residual buffer to prevent the presence of ethanol from interfering with subsequent reactions. DNA was eluted with 200 µl of elution buffer (Buffer AE).

The DNA from blood samples collected from cattle in western Turkey as well as from limit dilution of piroplasms in blood were extracted by the saponin lysis method as described in section 3.2.5.1. One blood sample of an infected calf was included as a negative sample for every 11 test samples during DNA extraction as a control for contamination.

7.2.3. Nested Polymerase Chain Reaction

A nested PCR was carried out to amplify *T. annulata* DNA in blood samples (McPherson *et al.*, 1993). Primers were designed from the gene encoding the 30 kDa merozoite surface antigen of *T. annulata* (Shiels *et al.*, 1995; Katzer *et al.*, 1998a). The first amplification of the nested PCR was carried out in a final volume of 50 µl using primers T3 and T5 amplifying a 924 bp fragment of the gene (Table 7.1). The reagents for the PCR were 0.5 µM each primer, 0.2mM each dNTP (Pharmacia

Biotech), 20 mM Tris-HCl (pH 8.55)*, 16 mM $(NH_4)_2$ SO_4*, 2.5 mM $MgCl_2$*, 150 µg/ml Bovine Serum Albumin* (*all contained in the 10X reaction buffer supplied with the *Taq* polymerase enzyme), 0.025U/µl *Taq* Polymerase (Ultrotaq DNA Polymerase, Thermometric Ltd.) and 2 µl test or control DNA sample in a final volume of 50 µl. The reaction solution in each tube was covered with sterile mineral oil (Sigma). Thin walled 200 µl tubes were used in an Omni Gene PCR thermal cycler (Hybaid). Cycling conditions were 94°C for 5 minutes followed by 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min. A final extension step of 72°C for 10 min was also used. In addition to test DNA samples, a positive control (DNA from a *T. annulata* infected cell line), a negative sample control (containing bovine DNA only) and a DNA negative control (5 µl Milli-Q water substituted for DNA) were included in the PCR reaction.

Primers Tams1F and Tspms1R were used in the second amplification of the nested PCR, amplifying a 785 bp fragment of the gene (Table 7.1). The reagents for the PCR were at the same final concentration as the first amplification in a 20 µl final volume. Two µl of DNA from the first amplification were used as template. Cycling conditions were identical to those described earlier for the first amplification.

Table 7.1. PCR primers from the 30 kDa merozoite surface antigen gene.

Primer	Sequence	Specificity
T5	5' ATT TAA ATC GCT CAC TAG TCT GC 3'	*Theileria* spp
T3	5' GAT AAG TTG TTA CGA ACAT GGG TTT 3'	*Theileria* spp
Tams1F	5' ATG CTG CAA ATG AGG AT 3'	*T. annulata*
Tspms1R	5' GGA CTG ATG AGA AGA CGA TGA G 3'	*Theileria* spp

The PCR products (10 µl) were separated on a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide and then used for Southern blotting (see section 3.2.5.3). In order to demonstrate that the PCR products were specific to the 30 kDa gene, a

20-mer sequence (5' ATC TGH CTG TGA CAT TTG HA3') from within the Tams-1 gene was used as a probe. The 20-mer oligonucleotide probe was labelled with DIG 3' End Labelling Kit according to the manufacturer's instruction (Boehringer Mannheim) and used in Southern blot hybridisation. For labelling, 100 pmoles of probe was mixed with 4 µl Tailing buffer (Vial 1; 1M potassium cacodylate, 125 mM Tris-HCl, 1.125 mg/ml BSA pH 6.6), 4µl $CoCl_2$ (25 mM), 1 µl DIG dd-UTP (1 mM Digoxigenin-11-dUTP), 1 µl terminal transferase (50 units/µl) and 80 mΩ water to 20 µl. The mixture was incubated at 37°C for 15 minutes and stored at –20°C until use.

Nylon blots were prehybridised in DIG Easy Hyb buffer (Boehringer Mannheim) at 40°C for 4 hours. Hybridisation was carried out overnight at 40°C in fresh buffer containing 30 pmoles DIG labelled DNA probe previously denatured by boiling for 10 minutes. DIG labelled DNA molecular weight marker XIV (100 base pair ladder, Boehringer Mannheim) was also added to the hybridisation buffer. Washes were performed twice in 2 x SSC, 0.1% SDS at room temperature for five minutes and twice in 0.5 x SSC, 0.1% SDS at 45°C. The detection of hybridised product was visualised with an immunological detection system using the DIG DNA Labelling and Detection kit (Boehringer Mannheim) according to the manufacturer's instructions as described in section 3.2.5.3.

7.2.4. Statistical analysis

The results obtained from field samples by the nested PCR, microscopic examination of Giemsa's stained blood and lymph node biopsy smears and isolation of macroschizont infected cells were analysed using the Chi-square distribution (Minitab, version 10.2).

7.3. RESULTS

7.3.1. Detection of *T. annulata* in Bovine Blood

T. annulata Gharb piroplasm infected blood with parasitaemia ranging from 1% at ten fold dilution steps to 10^{-9}% was used to test the sensitivity of the PCR. Figure 7.2 shows the results of the nested PCR and Southern blot hybridisation. The lowest concentration of piroplasms that could be detected was 10^{-5}% i.e. 1 piroplasm per 10^7 red blood cells, by both PCR (Figure 7.2A) and Southern blot hybridisation (Figure 7.2B) with DNA prepared using QIAamp Blood Kit. Sensitivity of the PCR was reduced to 10^{-4}% i.e. 1 piroplasm per 10^6 red blood cells, when Saponin lysis buffer was used to isolate DNA. A similar level of sensitivity was obtained using the Ankara stock of *T. annulata* (data not shown). This experiment was repeated with blood kept at –20°C after 6 months and the same result was obtained (data not shown).

7.3.2. Detection of Different Stocks of *T. annulata*

The nested PCR was performed using *T. annulata* stocks from different geographical areas in the range of the parasite and other stocks from one province in Turkey. Figure 7.3A indicates that the PCR detected all stocks tested. These results were confirmed by Southern blot hybridisation (Figure 7.3B).

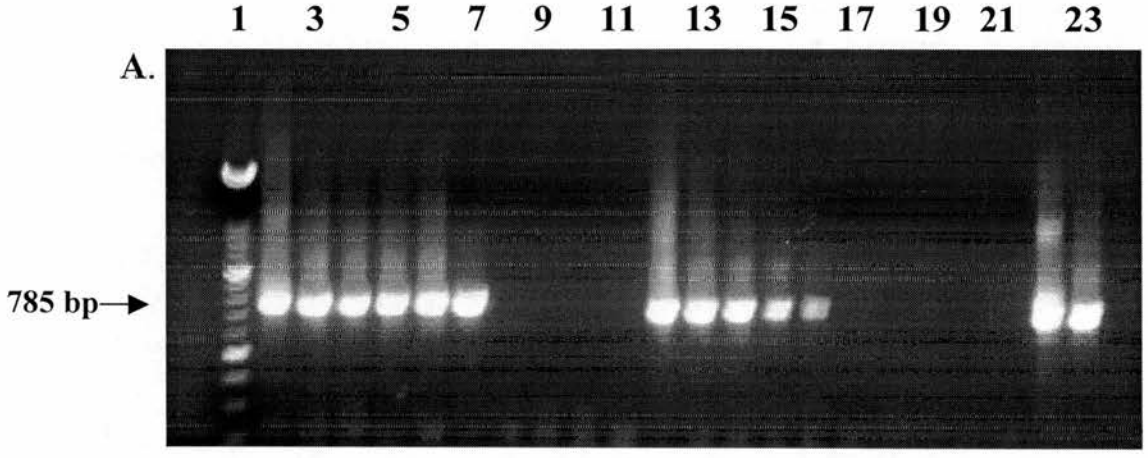
7.3.3. Cross-Reactions

DNA from parasites related to *T. annulata* was not amplified by this nested PCR. However, non-specific amplification occasionally occurred, possibly due to the prolonged number of cycles. These PCR products were shown to be non-specific by Southern blotting (Figure 7.4).

7.3.4. Field Survey around Aydin, Turkey

Figure 7.5 shows the PCR results in conjunction with the results of microscopic examination of blood smears, lymph node biopsy and isolation of macroschizont infected cells by cell culture. Blood smear examinations of 151 cattle from the province of Aydin revealed that 100 (66%) of cattle were positive for

Figure 7.2. Ethidium bromide-stained gel (A) and Southern blot (B) of the PCR to determine the level of sensitivity of the test using DNA extracted from *T. annulata* Gharb infected blood, serially diluted with uninfected bovine blood. DNA was extracted by both Qiagen kit and saponin lysis buffer. Lane 1: X1V Marker; lanes 2-11 show the DNA extraction by Qiagen blood kit; lanes 12-21 show the DNA extraction by saponin lysis buffer. Lanes 2-10 and 12-20: 1% piroplasm parasitaemia, decreasing in 10 fold dilution steps to 10^{-8}%; lane 11 and 21: uninfected bovine blood; Lane 22-23: DNA from *T. annulata* Ankara and Gharb cell lines; Lane 24: No DNA control.


1 3 5 7 9 11 13 15 17 19 21 23
A.
785 bp

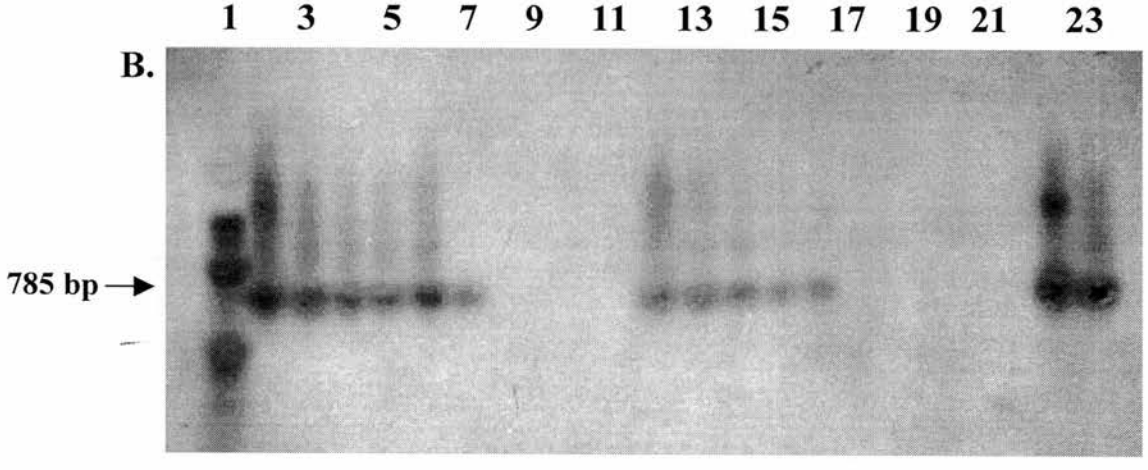
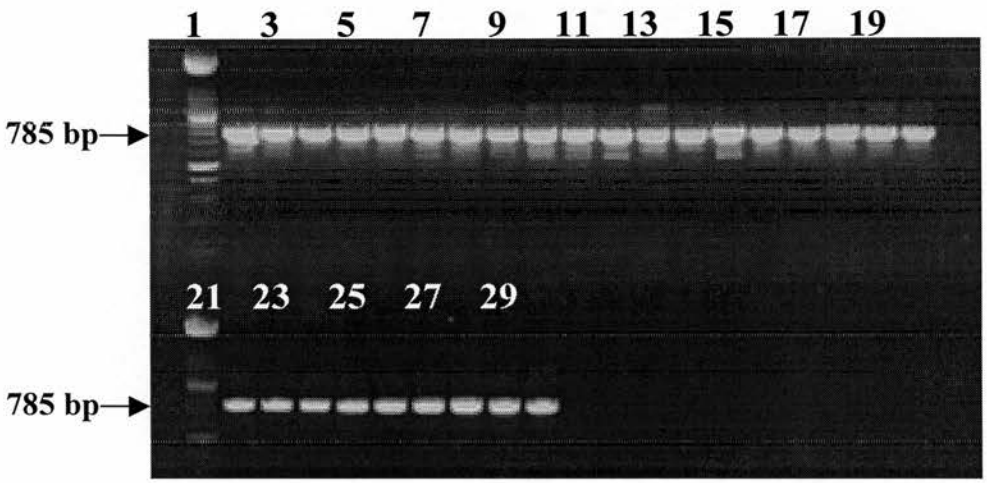

1 3 5 7 9 11 13 15 17 19 21 23
B.
785 bp

Figure 7.3. Ethidium bromide stained agarose gel (A) and Southern blot (b) of the PCR demonstrating the specificity of the test with cell lines of 28 different stocks of *T. annulata* across its geographical range. Top line: Lane 1 and 21: XIV Marker (Boehringer Mannheim); lanes 2 to 9: Caceres (Spain), Gharb (Morocco), Battan (Tunisia), Soba (Sudan), Tova (Israel), Ankara (Turkey), Razi (Iran), Hisar (India). The remaining lanes between 10 and 30 on the gel are isolates from different villages around Aydin, Turkey.


1 3 5 7 9 11 13 15 17 19
785 bp
21 23 25 27 29
785 bp

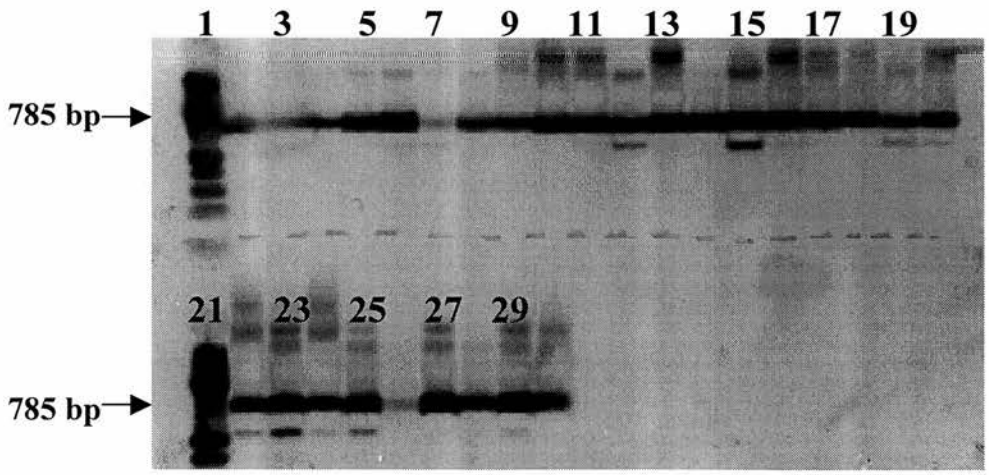
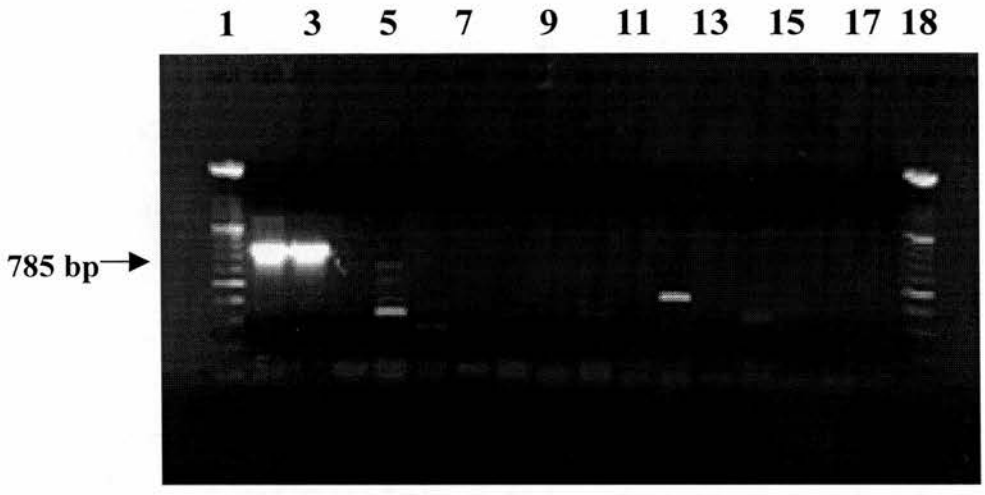

1 3 5 7 9 11 13 15 17 19
785 bp
21 23 25 27 29
785 bp

Figure 7. 4. Ethidium bromide stained agarose gel (A) and Southern blot (B) of the PCR demonstrating lack of cross-reactivity with closely related parasites. Lane 1: Marker, lane 2: *T. annulata* Gharb cell line, lane 3: *T. annulata* Razi cell line, lane 4: *T. hirci* – Lahr cell line, lane 5: *T. lestoquardi* Shiraz cell line, lane 6: *B. bigemina* (Kenya), lane 7: *B. bovis* (Mexico), lane 8: *B. divergens* (Stormont), lane 9: *B. equi* USDA, lane 10: *B. equi* Onderstepoort, lane 11: *B. equi* Kwa Zulu, lane 12: *T. buffeli* Australia, lanc 13: Bovinc PBM, lane 14: Sheep PBM, lane 15: Horse whole blood, lane 16: Adult tick, lane 17: No DNA.


1 3 5 7 9 11 13 15 17 18
785 bp

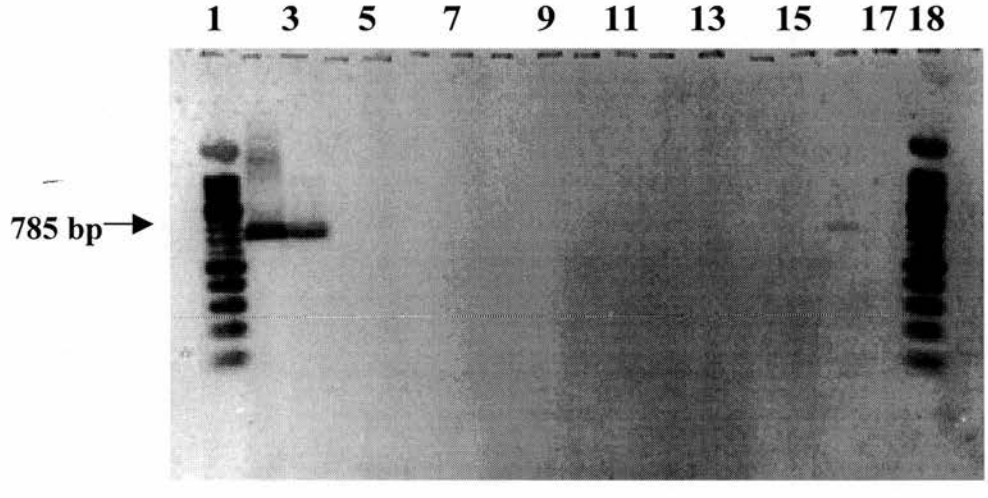
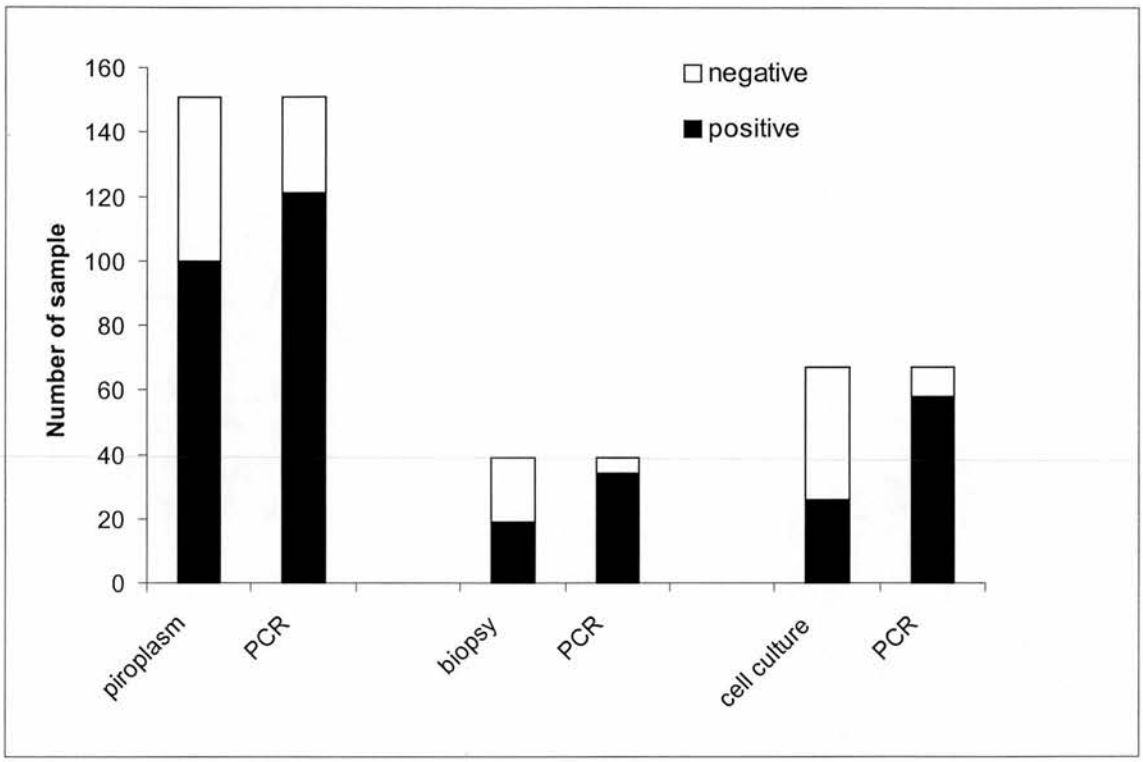

1 3 5 7 9 11 13 15 17 18
785 bp

Figure 7.5. Results of microscopic examination of thin blood, lymph node biopsy smears for the detection of the presence of piroplasms and macroschizont respectively, and establishment of macroschizont infected cells from cattle. The results are given in comparison to PCR results of the same samples.


160
140
120
100
80
60
40
20
0
Number of sample
negative
positive
piroplasm
PCR
biopsy
PCR
cell culture
PCR

piroplasms, which based on morphology of the piroplasms and clinical symptoms of the cattle, were assumed to be *T. annulata*. There was a concurrent clinical infection of *Anaplasma marginale* in one animal. Two animals were infected with *A. marginale* alone and another two with *Babesia* spp. alone. These animals were negative for *T. annulata* using the PCR. In total 121 of 151 animal tested were positive in the *T. annulata* PCR. *T. annulata* piroplasms were seen in only 100 of these 151 cattle. Three of the 21 PCR positive, blood smear negative animals were positive by lymph node biopsy. Macroschizonts were detected in 20 out of 39 cattle sampled by lymph node biopsy, 34 of these 39 cattle were positive by PCR (Figure 7.5).

Sixty-seven blood samples from animals showing symptoms of theileriosis were used to establish *T. annulata* macroschizont-infected cell lines. Several problems occurred at the beginning of the experiment. During the first two weeks, flasks with the cap loose were placed into a sandwich box and 5% CO_2 was supplied using a fire extinguisher. Bacterial contamination was found in many of the cell cultures and fifteen cell cultures were discarded because of bacterial contamination. Subsequently 5% CO_2 was omitted and the flask caps were tightened. A total of 26 macroschizont infected cell lines were isolated from 7 different districts of Aydin. Two of these cultures showed mixed infections with *Trypanosoma theileri*; another was infected with *Try. theileri* only. One of the cultures co-infected with *Try. theileri* was successfully treated with amphotericin-B (Fungizon, Gibco)at a dose of 2.5 µl/ml for two days in the RPMI-1640 medium while the trypanosomes disappeared from the second cell line during the course of passage. Because of these problems, it was not possible to compare the results of the cell culture isolation and PCR, but all *Theileria* positive cell cultures were positive for PCR.

Statistical analysis using X^2 indicated that PCR was significantly more sensitive than all other diagnostic tests performed in the current study, i.e. microscopic examination of piroplasms ($p = 0.006$), lymph node biopsy ($p = 0.000$) and cell culture ($p= 0.000$).

7.4. DISCUSSION

The aim of the current study was to develop a sensitive and specific PCR that can be used as a diagnostic tool to detect the carrier state of theileriosis. The nested PCR using primers derived from the gene encoding the 30 kDa merozoite surface antigen (Tams-1) of *T. annulata* is demonstrated to amplify parasite DNA using blood samples from animals exhibiting low piroplasm parasitaemia.

The nested PCR method has proved to be 100 times more sensitive than searching 200 fields in high power light microscopy assuming that cattle have 10^7 red blood cells per µl of blood, detecting piroplasms at 10^{-5} % i.e. 1 piroplasm in 10^7 red blood cells. This is equivalent to the detection of 1 piroplasm in 1 µl of blood. 200 fields of light microscopy at 1000 x magnification corresponds approximately to 10^5 red blood cells. In the present study, 50 fields of microscopy were examined as a result of which the comparative sensitivity of the nested PCR is increased a further 4 times.

Experiments performed with samples collected from the field also demonstrated that the sensitivity of the nested PCR was significantly higher both in detecting the carrier state and in clinical diagnosis of the disease than that obtained by conventional diagnostic tests (microscopic examination of Giemsa's stained blood and lymph node biopsy smears or isolation of macroschizont infected cell lines). It should be noted that PCR performed with DNA extracted using the QIAamp blood kit was more sensitive than that using saponin lysis buffer indicating that the sensitivity of the PCR is affected by the method of DNA extraction. In any case, these results would strongly suggest that the carrier state of animals infected with *T. annulata* could be diagnosed using the PCR with a greater degree of sensitivity.

The sensitivity of the PCR was first tested with a number of different stocks of *T. annulata* of widely diverse geographical origin since it was previously demonstrated that the Tams-1 protein is very polymorphic (Katzer *et al.*, 1998a). Over 30 stocks of the parasite collected from ten countries across a wide range of *T. annulata* distribution and from Aydin province of Turkey could be detected with the nested PCR. All these parasites have been shown to be phenotypically diverse by glucose phosphate isomerase polymorphism (Melrose *et al.*, 1980); E. Kirvar pers.

comm.). It appears from these observations that the nested PCR can detect *T. annulata* across its geographical range.

A PCR-based methodology using primers N516 and N517, also derived from the Tams-1 gene, was previously developed by d'Oliveira *et al.* (1995; 1997b). The sensitivity of the PCR developed by d'Oliveira *et al.* (1995) was 2-3 piroplasms per µl (10^7 red blood cells). However, the same level of sensitivity could not be obtained in our laboratory and the sensitivity of PCR obtained using primers N516 and N517 was only 1 piroplasm in 10^5 cells (data not shown). Additionally, the amplification of DNA from some of the *T. annulata* stocks isolated from Aydin province of Turkey using the primers N516 and N517 was inconsistent (data not shown). This substantial differences in observed sensitivity and specificity of PCRs described here and by d'Oliveira *et al.* (1995, 1997) might be ascribed to differences in the primers used in the respective methods.

Microscopic examination of Giemsa's stained blood smears is the traditional method used for the detection of *T. annulata* piroplasms. Nevertheless, it is not possible to distinguish between *T. annulata* species on the bases of morphological differences, e.g. proportion of annular vs. pyriform piroplasms (*T. annulata* vs. *T. parva*), and presence of 'bars' and 'veils' (*T. buffeli*) (Norval *et al.*, 1992). The results showed that the nested PCR described in the current study could distinguish *T. annulata* from other bovine *Theileria* species tested. This is important for epidemiological studies in areas where the distribution of these parasites overlaps. The nested PCR could also distinguish *T. annulata* from *Babesia spp.* and *Anaplāsma* spp. Since mixed infections with these parasites occur very often in cattle, the nested PCR should prove useful for differential diagnosis.

Additionally, and most importantly, the nested PCR also distinguished the closely related parasites of sheep, *T. lestoquardi (=hirci)* and of horses, *Babesia (=Theileria) equi,* from *T. annulata*. All three parasites, *T. annulata, T. lestoquardi* and *Babesia equi* are all transmitted by *Hyalomma* ticks (Hooshmand-Rad and Hawa, 1973; Robinson, 1982; Friedhoff, 1988). Although it was previously demonstrated that *T. lestoquardi* cannot infect cattle (Brown *et al.*, 1998a; Leemans *et al.*, 1999b), the accurate identification of these species in ticks is important to assess the potential threat of *T. annulata* in a particular area and to gather

epidemiological data on tick infection rates. The presence of *Theileria* in ticks is traditionally determined by staining dissected salivary glands with methyl green pyronin (Walker and McKellar, 1983). Despite of its relative simplicity, however, this method can only be performed on live ticks and does not allow differentiation of these parasites (Walker and McKellar, 1983; Kirvar *et al.*, 1998). The inner set of primers used in the current study was also demonstrated to be effective in detecting *T. annulata* using DNA from infected vector ticks (Kirvar *et al.*, 1999).

Taken together, the nested PCR described in the current study is shown to be both sensitive and specific and would be valuable in epidemiological studies of *T. annulata* for both carrier animals and vector ticks. Such studies are needed for an accurate assessment of theileriosis in risk areas, a prerequisite for effectively targeting of vaccines for the control of tropical theileriosis.

CHAPTER EIGHT

GENERAL DISCUSSION

Tropical theileriosis, a disease of cattle caused by infection with the protozoan parasite *T. annulata*, presents an important constraint to livestock development in Asia, north Africa and southern Europe (Purnell, 1978). The control of tropical theileriosis in many countries has primarily been carried out by vaccination with attenuated macroschizont infected high passage cell lines (Pipano, 1976; Hashemi-Fesharki, 1988; Onar, 1989; Ouhelli, 1991; Singh, 1991; Zablotsky, 1991). Targeting the available vaccine where it is most needed and monitoring to determine whether vaccinated animals are withstanding sporozoite challenge through infected tick in the field requires comprehensive epidemiological disease surveys. Highly sensitive and specific diagnostic techniques are necessary to detect the presence of parasite or antibodies which recognise parasite antigens. To date the most common method used to monitor the response to theilerial vaccines and natural infection is the IFAT using both macroschizont and piroplasm antigens (Pipano and Cahana, 1969; Anon, 1997). However, IFAT does not give any indication of which animals have been vaccinated or which animals are naturally infected through infected ticks. This is because the presence of common antigens is present in different life cycle stages of the parasite in the vertebrate host.

The primary objective of this study was to develop sensitive and specific diagnostic tests for *T. annulata* infection in particular to distinguish animals vaccinated with attenuated macroschizont infected cells from naturally infected animals and to detect sensitively and specifically the carrier state in cattle. The work was carried out in the following stages: (i) design and conduct animal experiments to produce material for development and evaluation of stage specific ELISAs and highly sensitive and specific PCR, (ii) clone a macroschizont gene of *T. annulata* and express it as recombinant protein to use in ELISA, (iii) establish the diagnostic sensitivity and specificity of the Tamr-1 and macroschizont ELISAs for detection of antibodies against *T. annulata*, (iv) assess the ability of Tamr-1 and macroschizont

ELISAs to distinguish vaccinated animals from those naturally infected, (v) develop a highly sensitive and specific PCR methodology using *T. annulata* specific primers from the gene encoding 30 kDa merozoite surface antigen (Tams1) to detect low level infections in carrier animals (vi) evaluate PCR on experimental material raised in (i).

Three main groups of calves were infected with *T. annulata* Ankara at different parasite stages and at different levels of attenuation, i.e. sporozoites (Group 1), a non attenuated low passage cell line (Groups 2A and 2B), an attenuated high passage cell line (Ankara/Pendik) (Groups 3A and 3B). The calves were challenged one or seven months after primary infection with a heterologous sporozoite stabilate, *T. annulata* Gharb. The severity of reaction to infection and challenge were categorised as severe, moderate and mild according to the results obtained from clinical, parasitological and haematological parameters (Anon, 1989). Reaction to primary infection was different in each group. All calves in Group 1 underwent a severe reaction to primary infection. Calves in Groups 2A and 2B showed a wide range of reactions from severe to mild to primary infection. In contrast, all calves in Groups 3A and 3B underwent only mild reactions. Following challenge all animals in Groups 1, 2A and 2B exhibited solid immunity to challenge response 1 month or 7 months after primary infection. Following challenge, these calves, underwent a transient temperature reaction with no or only slight decrease in WBC. Only one calf in Group 2B showed a long period of pyrexia, but haematological and parasitological results suggested that this temperature response might not have been related to the challenge. However, calves in Group 3A and 3B showed moderate or severe reactions to challenge. In sum, immunity stimulated by the Ankara/Pendik cell line protected three out of four animals one month following vaccination (Group 3B) and two out of four 7 months following vaccination (Group 3A). The sporozoite challenge infection of the control group was lethal and despite chemotherapy two calves died.

The carrier state of animals was determined by microscopic detection of piroplasms, cell culture isolation of macroschizonts infected cells from peripheral blood and a PCR method (Ilhan, 1995) using primers from ssu rRNA (Allsopp *et al.*, 1993). Animals infected with sporozoites or the low passage cell line were shown to

be persistent carriers following infection by PCR and the presence of piroplasms in blood smears. In addition, cell cultures could be established more frequently from these animals than animals immunised with the high passage cell line. Animals infected with the high passage cell line were rarely positive by PCR, or cell culture and only very rare piroplasm-like organisms were detected infrequently in these animals before challenge.

Antibody responses of these animals were monitored by the IFAT using macroschizont and piroplasm antigens as a conventional test and by the IgG and IgM ELISAs using three recombinant antigens, Tamr-1 (merozoite rhoptry), NC10-Ssp13 (macroschizont) and Tash-2 (macroschizont and piroplasm). All calves sero-converted with IFAT using both macroschizont and piroplasm antigens. The macroschizont IFAT result showed a relatively similar pattern in all calves. Differences in antibody responses were revealed by the piroplasm IFAT and the Tamr-1 and Tash-2 ELISAs. Anti-piroplasm antibodies were detected by IFAT even though piroplasms were not seen in the calves immunised with the high passage cell line. However, the antibody levels and duration of responses was lower and shorter than those after infection with the sporozoites and the low passage cell line. Similarly, no antibody response or very slight increase in the antibody levels were observed using the Tamr-1 and Tash-2 ELISAs in animals immunised with the high passage cell line.

Taken together these observations indicate a correlation between the primary infection, the immune response to challenge, the carrier status and antibody responses of these animals. Calves which showed solid immunity to challenge (i) exhibited a moderate to severe clinical reactions to primary infection, (ii) were persistent carriers and (iii) showed a significant increase in antibody levels in the IgG Tamr-1 and Tash-2 ELISAs. In contrast, where protection against challenge was poor or moderate, calves (i) exhibited inapparent or mild clinical and parasitological reactions to primary infection, (ii) were intermittent carriers of the parasite, and (iii) showed a low level and short duration of antibody responses to piroplasm IFAT and no or slight increase of antibody levels in the Tamr-1 and Tash-2 ELISAs.

The reason why animals immunised with attenuated cell lines were not well protected from challenge is of considerable interest and potential relevance as such

cell lines are used as vaccines in Turkey. Several factors are considered to be involved in the induction of immunity by macroschizont-infected cell cultures. These factors include (i) the level of attenuation of a cell culture, (ii) transfer of macroschizonts from inoculated cells into recipient's cells with establishment, multiplication and dissemination, (iii) induction of a carrier state with resultant premunity.

The aim of attenuation of the parasite is to reduce its virulence while maintaining its immunogenicity and infectivity (Pipano, 1977; Pipano, 1989b). Complete attenuation is accompanied by the loss of ability to produce merozoites thus piroplasms (Pipano, 1977). Different field isolates of *T. annulata* require different periods in culture before reaching complete attenuation (Hooshmand-Rad, 1973). However, prolonged passages appear to reduce infectivity and immunogenicity (Brown, 1990). Therefore, the Ankara/Pendik vaccine cell line was subcultured more than 300 times over 3 years.

Attenuated macroschizonts grown in culture may undergo only a limited number of replication cycles when inoculated into cattle (Pipano, 1977) which may result in the poor transfer of the parasite into the recipient's reticulo-endothelial cells. It has been demonstrated that attenuated *T. annulata* Ode cells disseminate very poorly compared to virulent parent cell lines in *scid* mice (Somerville *et al.*, 1998a). The degree of dissemination was correlated with a marked reduction in matrix metalloproteinases (MMP) which are known to play an important role in metastasis (Sier *et al.*, 1996). It is believed that to achieve immunity with macroschizont infected cell culture, the parasite must establish and replicate within the recipient's mononuclear cells (Brown *et al.*, 1978a; Brown and Gray, 1981). Therefore, excessively attenuated macroschizont infected cells may not reach a sufficient quantity needed to induce immunity and may not induce a carrier state with resultant premunity or co-infectious immunity as defined by Sergent (1963).

Although the antibody detected by a serological test is not necessarily an indication of the immunity that has developed against infection, the presence of antibody may indicate either multiplication of macroschizonts with or without the presence of piroplasms. In another experiment, calves that were infected with sporozoites were immune to a heterologous challenge three years after primary

infection (Preston and Brown, unpublished). These animals were consistent piroplasm carriers until the challenge and antibodies could be detected 3 years following primary infection with the Tash-2 ELISA. The presence of piroplasms and detection of anti-macroschizont antibodies would indicate that calves infected with sporozoites are carriers of both macroschizonts and piroplasms. This is contrast to the classical definition of a carrier of *T. annulata* that is the ability of an infected and recovered animal to serve as a reservoir of infection for vector ticks which are able to transmit the parasite to susceptible hosts (Young *et al.*, 1986). Currently the carrier status is thought to be maintained by the persistent and slow division of macroschizonts giving rise to piroplasms (Norval *et al.*, 1992).

In the current study, immunity stimulated by the Ankara/Pendik vaccine cell line protected three out of four animals one month following vaccination which was only marginally better than the protection observed (2/4) 7 months following immunisation. Cattle immunised in the field where the prevalence of disease is high are likely to be exposed to field, i.e. tick/sporozoite challenge during the first disease season and become immune for long periods. However, if animals pass the first disease season without challenge, they may become susceptible to disease in following years. Therefore, whether repeated vaccination may be required will depend on the level of field challenge and endemic stability in the region. However, re-immunisation with the same cell line may not boost immunity because of a graft rejection type response (Nichani *et al.*, 1997a; Nichani *et al.*, 1997b).

Several approaches were tested in order to obtain a macroschizont stage specific antigen for use in an ELISA to detect anti-theilerial antibodies even in vaccinated animals. Screening of a cDNA library of *T. annulata* with the QP gene, (Baylis *et al.*, 1993) which is equivalent to PIM, a polymorphic immunodominant macroschizont gene of *T. parva* (Toye *et al.*, 1991; 1995a; 1995b), resulted in isolation of three genes, NC1, NC2 and NC10. Only the NC1 and NC10 genes were sequenced and the sequence analysis showed that there is not high homology between the QP gene and gene represented by the cDNA clones. The region of similarity between the predicted amino acid sequences of cDNA clones and the QP/PIM gene was found to be over the Gln-Pro and Glu-Pro regions. However, the NC10 gene shows tetrapeptide repeats with QP repeats in its central region and these

repeats are similar to the repeats observed in the PIM gene sequence (Baylis *et al.*, 1993; Toye *et al.*, 1995b). Similar motifs were also demonstrated for the p150 gene of *T. parva* (Skilton *et al.*, 1998). Therefore, these motifs and sequence homologies may represent a common ancestry for NC10, PIM and p150 genes.

Northern blot analysis of the cDNA clones demonstrated that genes represented by clones NC1, NC2 and NC10 were differentially expressed by different life cycle stages of the parasite. The NC1 and NC2 were expressed mainly by the sporozoite stage and clearly down-regulated at the piroplasm stage. In contrast, the NC10 gene was expressed mainly at the macroschizont stage of the parasite life cycle. The QP probe, however, detected at least three mRNA species of *T. annulata*. At the stringency used a higher level of homology would be expected for detection of the same RNA species by these probes. Additionally, both QP and NC2 probes do not detect the same RFLP's on Southern blots at 60°C stringency. Thus, it is most likely that the mRNA species detected by the NC1, NC2 and NC10 genes is not the same mRNA species that is detected by QP protein.

The results of sequence, Southern and northern blot analyses suggest that none of the genes isolated from the *T. annulata* cDNA library have real homology to the QP/PIM gene. There is a possibility that there is a gene(s) in the *T. annulata* genome with a higher homology to the QP/PIM gene. On the other hand, the NC10 gene is QP/PIM-like in terms of the tetrapeptide repeats with QP motifs and thus the encoded protein could be a functional homologue of the QP/PIM gene. Additionally, since the QP/PIM gene is highly polymorphic among *T. parva* stocks (Toye *et al.*, 1995a; 1995b), the presence of selection for diversity in this antigen in one or both species may have resulted in the low level of homology displayed between PIM and the *T. annulata* cDNA clones.

Since both NC1 and NC10 genes were expressed by the macroschizont stage of the parasite, attempts were made to produce recombinant proteins from these genes. The expression of NC1 gene fragments was toxic to the strains of *E. coli* used. However, a 1.2 kb fragment of NC10 gene, NC10-Ssp13, was successfully expressed as a His-tagged fusion protein using both pGex and pQE vector systems and the expression product was immunogenic as it reacted with immune bovine sera. Results of western blot analysis demonstrated that several polypeptides from macroschizont

infected cells of different *T. annulata* stocks reacted with the rabbit anti-NC10-Ssp13 serum. The detection of several polypeptides could be due to fact that: (i) the NC10-Ssp13 fusion protein contains repeated amino acid motifs and therefore, it is possible that some of the bands detected were due to cross reactivity of the anti-serum with similar repeats within different proteins as shown for other parasites (Anders, 1986; Skilton *et al.*, 1998); or (ii) there could be more than one related gene for NC10 gene in the *T. annulata* genome as two mRNA species were detected by the NC10 cDNA probe in northern blot analysis and the anti-NC10 sera detected proteins on both macroschizont and nucleus of the host cells.

The results of IFAT using anti-NC10-Ssp13 sera against macroschizont infected cells were very interesting. The immunofluorescence reactivity was detected against both macroschizonts and the host cell nucleus. Host nuclear reactivity was scattered over the region of nuclear envelope. The staining of the host nucleus was specific to cells in interphase as no staining was observed in cells undergoing mitotic division. These results indicate that the protein detected on the host nucleus may be related to infection and immortalisation of the host cell by the parasite. At present, identification of mechanisms related to induction of host cell proliferation by *Theileria* is limited and not fully understood (reviewed by Chaussepied and Langsley, 1996; Dobbelaere and Heussler, 1999). Recently, it was reported that a parasite molecule, TashAT, is transported to the host cell nucleus and could be involved in regulating proliferation and/or host cell gene expression (Swan *et al.*, 1999). The staining pattern of macroschizont infected cells obtained by the TashAT gene was similar to that obtained by NC10 gene but not identical. Staining with anti-TashAT antibody has also been observed in cells during mitosis (Shiels, Stern and Swan, unpublished). Therefore, while the TashAT and NC10 genes may both be involved in the control of host cell proliferation it is possibly they perform distinct complementary functions related to a location either within or on the surface of the host nucleus.

Further studies that would clarify several aspects of the results obtained here include (i) complete the sequence of the 5' end of the NC1 and NC10 genes, (ii) carry out further Southern blot analysis of NC10 gene at high stringency to confirm the gene is single copy, (iii) investigate the fate of the protein during mitosis using

electron microscopic examination (iv) carry out transfection studies to confirm whether the NC10 antigen is responsible for the fluorescence pattern described in the current study or if is this due to a related parasite antigen or cross recognition of a host polypeptide.

Three recombinant antigens expressed in different life cycle stages of the parasite, Tamr-1 (merozoite/piroplasm), NC10-Ssp13 (macroschizont) and Tash-2 (macroschizont/piroplasm) were demonstrated to be antigenic by western blot analysis using serum from the majority of animals infected with *T. annulata* sporozoites and low passage cell lines. Subsequently, these antigens were used in indirect ELISAs. According to this scheme, if the Tamr-1 ELISA and either the NC10-Ssp13 or the Tash-2 ELISA are positive, it can be concluded that the animal has been infected or challenged with sporozoites regardless of whether it had been immunised. On the other hand, a negative result with the Tamr-1 ELISA and a positive result with the NC10-Ssp13 or Tash-2 ELISA would indicate that the animal was only immunised with an attenuated macroschizont infected cell line, and not yet exposed to field challenge with tick transmitted sporozoites.

The indirect ELISA format was selected to detect both IgG and IgM antibodies against *T. annulata* infection. International guidelines were adapted for the IgG Tamr-1 ELISA (Wright *et al.*, 1993). In particular, ODs were expressed as a positivity index (percent positivity, PP) of the high positive control (C++) on each ELISA plate to minimise the effects of plate to plate and day to day variation. In addition control limits were determined for four controls (C++, C+, C- and Cc) and used to accept and reject each plate. Recently, computer software packages such as Shewhard-CUSUM (Blacksell *et al.*, 1996) have been increasingly used to monitor ELISA performance. It was suggested by Blacksell *et al.* (1996) that at least ten tests should be conducted to provide reference control data. In the current study for the IgG Tamr-1 ELISA, six replicate tests were conducted to establish control limits to accept or reject a plate. Quality control limits are an essential feature of ELISAs because of their inherent variability. Various measures have been recommended to minimise such variations for example: 1) the use of large reagent batches stored under optimal conditions, 2) technical training of operators, 3) good laboratory management (Kemeny, 1991).

The categorisation of animals as positive and negative by ELISA is dependent on the cut-off value chosen. The cut-off value for the Tamr-1 ELISA was established using a frequency distribution of results from positive and negative animals (Wright *et al.*, 1993), and a method of two graphs receiver operating characteristic (TG-ROC) analysis (Greiner *et al.*, 1995) which graphically represents the effect of different cut-off values on sensitivity and specificity. The TG-ROC analysis could be carried out using a computer programme, which is public domain software (Greiner, 1995). In many studies, TG-ROC was used in evaluation of sensitivity and specificity of ELISA (Greiner *et al.*, 1997; Gubbels *et al.*, 1999a; Mboloi *et al.*, 1999). However, in the current study TG-ROC were empirically prepared using the cumulative frequency distribution of negative (CFD_{neg}) and the inverse positive (CFD_{pos}) sample (Greiner *et al.*, 1995) using cut-off values of 0 - 100 PP at 1 PP intervals. Cut-off values of 14 and 18 PP were chosen as the values that gave the best estimates of sensitivity and specificity for the IgG Tamr-1 ELISA.

By using 209 calves experimentally infected with *T. annulata* the diagnostic sensitivity of the IgG Tamr-1 ELISA was 78.5% and 72.7% at 14 PP and 18 PP cut-off values, respectively. Both of the values were substantially lower than the sensitivity obtained with these serum samples using the IFAT piroplasm antigen (100%). However, the specificity of the test determined using 188 negative cattle was 95.2% and 98.4% at 14 PP and 18 PP cut-off values, respectively which is very close to that obtained by IFAT (100%). Here, only *T. annulata* positive and negative animals were used which did not pick up on the non-specificity of the IFAT for example. Although the IFAT is more reliable than the Tamr-1 ELISA, the IgG Tamr-1 ELISA could be very useful in large-scale sero-epidemiological studies, as it is less laborious than IFAT.

In order to assess the increase in antibody levels of individual calves infected with either *T. annulata* Ankara sporozoites or the low passage cell line or immunised with the high passage cell line the cut-off values for the NC10-Ssp13 and Tash-2 ELISAs were used as twice the absorbance value of pre-infection serum for each calf (de Savigny and Voller, 1980). The IgG and IgM ELISAs using the three recombinant antigens, Tamr-1, NC10-Ssp13 and Tash2, were analysed to assess their ability to distinguish vaccinated animals from those naturally infected and to

determine their value as diagnostic tools for epidemiological studies. Results were compared with the macroschizont and piroplasm IFAT. All three recombinant antigens detected antibody responses in calves infected with either sporozoites or the low passage cell line. The earliest antibody detection was observed with IFAT (between days 28-35), then the Tamr1 (between days 28-56), NC10-Ssp13 (day 84) and Tash2 (by day 84) antigens. The IgG Tamr-1 ELISA detected antibodies for over 7 months following the primary infection and over a year after challenge. On the other hand, both the IgG NC10-Ssp13 and Tash-2 ELISAs detected antibodies for three years even in absence of challenge. All the animals 'naturally' infected with sporozoites were piroplasm carriers for three years, implying the persistent presence of macroschizonts and their differentiation to merozoites/ piroplasms.

Effectively, no piroplasms were detected by examination of Giemsa's stained blood smears from animals immunised with the high passage cell line. These results confirmed the previous reports that Ankara/Pendik vaccine cell line does not produce piroplasm *in vivo* (Ozkoc and Pipano, 1981; Onar, 1989). As expected, no antibody responses were detected in these animals using the Tamr-1 ELISA indicating that this antigen could be used to distinguish vaccinated animals from naturally infected or challenged animals. Antibody responses were detected in calves immunised with the high passage cell line (Group 3A) with the both the IgG NC10-Ssp13 and Tash-2 ELISAs. However, antibody levels were very low and detected even later (day 111) than those in animals infected with sporozoites (day 84).

Taken together, the results indicated that ELISAs using the Tamr-1, NC10-Ssp13 or Tash-2 recombinant antigens cannot be used to distinguish animals vaccinated with the attenuated high passage cell line from those that are naturally infected under the standardised conditions described here. Firstly, the sensitivity obtained with the Tamr-1 ELISA is insufficient. Secondly, although the antibody responses of animals were readily detected three months after infection with sporozoites with the IgG NC10-Ssp13 and especially Tash-2 ELISAs the antibody levels were very close to pre-infection OD values in animals vaccinated with the high passage cell line.

Late detection of antibody in animals infected with sporozoites or immunised with the high passage cell line could be due to the fact that antibodies against *T.*

annulata may have low affinity to the Tash-2 antigen. The detection of low affinity antibodies could be improve by changing antigen concentrations, serum dilutions and washing conditions (Butler, 1988; Devey and Steward, 1988).

Another approach to discriminate vaccinated animals from naturally infected animals would be to label the cell line vaccines with a marker as described for the rinderpest virus (Baron and Barrett, 1997) and *Salmonella* live vaccine strains (Beyer and Bohm, 1996). In this way, it would be possible to monitor whether an animal has been vaccinated effectively i.e. the vaccine 'take'. The infection or challenge of animals with sporozoites could be then monitored by either the Tamr-1 or Tash-2 ELISAs.

Another aspect of the current study was to develop a sensitive and specific PCR method to detect low level infections in carrier animals. The nested PCR using primers derived from the gene encoding the 30 kDa merozoite surface antigen (Tams1) of *T. annulata* was able to amplify parasite DNA from blood exhibiting 10^{-5}% parasitaemia, i.e. 1 piroplasm in 1 µl blood. This is 100 x more sensitive than examining 200 fields by light microscopy at 1000 x magnification. The nested PCR was also more sensitive in detecting acute infections than isolation of macroschizont infected cells from peripheral blood of infected animals by cell culture and than the examination of Giemsa's stained lymph node biopsy smears. Additionally, the nested PCR is proven to be a specifically detected *T. annulata* DNA making it a better test than other diagnostic tests such as IFAT, ELISA which cross-react with related parasites.

In conclusion, even though the ELISAs developed here failed to distinguish vaccinated animals from naturally infected animals both Tamr-1 and Tash-2 ELISA could be powerful diagnostic tools. Both the Tamr-1 and Tash-2 ELISAs have the potential for use in epidemiological studies. However, further modification of the ELISAs is required to improve their sensitivities. The nested PCR is very sensitive and specific and would be valuable in epidemiological studies of *T. annulata* for both carrier animals and vector ticks. Such studies are not only needed for an accurate assessment of theileriosis in risk areas, but are also a prerequisite for effectively targeting of vaccines for the control of tropical theileriosis.

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